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Influenza entry pathways in polarized MDCK cells

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ABSTRACT

In non-polarized cell culture models, influenza virus has been shown to enter host cells via multiple endocytic pathways, including classical clathrin-mediated endocytic routes (CME), clathrin- and caveolae-independent routes and macropinocytosis. However, little is known about the entry route of influenza virus in differentiated epithelia, *in vivo* site of infection for influenza virus. Here, we show that in polarized Madin–Darby canine kidney type II (MDCK II) cells, influenza virus has a specific utilization of the clathrin-mediated endocytic pathway and requires Eps15 for host cell entry.

1. Introduction

Most animal viruses take advantage of the diverse cellular endocytic pathways to gain access to the host cells [1–3]. After virus binding to host cell receptors and being endocytosed, viruses can initiate replication only once they penetrate or fuse with the endosomal membrane [4]. A role of dynamin-dependent endocytosis for influenza virus entry was first demonstrated by examining the effect of dominant-negative (DN) dynamin, a small GTPase mediating the scission of clathrin-coated vesicles from the plasma membrane, on influenza virus entry in Mv-1 lung cells [5]. Studies from our group also showed that the infectivity of influenza viruses in cells expressing a DN mutant of Eps15 (epidermal growth factor receptor pathway substrate 15), which inhibits clathrin-dependent endocytosis specifically, was not impaired in HeLa cells [6]. When chemical inhibitors as well as DN mutant of caveolin-1 were used to disrupt caveolae-dependent endocytosis in host cells, influenza infectivity was retained as compared to that in untreated cells [6]. These observations led to the realization that non-clathrin-dependent, non-caveolae-dependent endocytic pathways exist for influenza virus entry, in addition to the classical clathrin-dependent pathway. More recently, with detailed dissection of influenza entry pathways independent of dynamin using pharmacological inhibitors, de Vries et al. discovered that influenza virus can utilize a macropinocytosis-like route in many cell types [7]. With the recent advancements of live cell imaging, individual influenza particles can be tracked in real time without disruption of endocytic pathways, a novel technique in discovering redundant or parallel endocytic pathways. With the new techniques, epsin1 (Epn1), an adaptor protein that interacts with clathrin, AP2 adaptors, and Eps15 in clathrin-coated pits, was demonstrated to be an influenza cargo-specific adaptor for entry via the clathrin-mediated endocytic (CME) pathway in BSC-1 cells [8]. Examining the dynamics of the endocytic uptake also led to the conclusion that influenza viruses exploit different pathways with the same efficiency, and these non-classical, less-characterized pathways do not act as alternative pathways for influenza virus entry.

Polarized, simple epithelial cells have a plasma membrane that is separated by tight junctions into two clearly distinct domains: the apical domain facing the tract lumen and the basolateral surface facing the extracellular matrix [9]. Cellular actin and the microtubule network, as well as an array of cellular proteins, participate in the organization and maintenance of cell polarity. It is well recognized that influenza enters and buds from apical domain of polarized MDCK cells [10]. Previous studies from our laboratory have demonstrated that actin microfilaments play different roles in influenza virus infection in polarized epithelial cells compared to non-polarized cells [11]. In contrast to their dispensable role in viral infection of non-polarized cells, intact actin filaments are obligatory for influenza virus infection in polarized epithelial cells. Since there are significant differences between polarized and non-polarized cells with regard to receptor distribution, cytoskeletal structure, trafficking events, and mechanism of endocytosis, it is possible that our current knowledge of influenza virus entry in non-polarized cells, such as HeLa, MDCK, BSC-1 cells, does not completely apply to *in vivo* viral infection, which is initiated at the differentiated airway epithelial cells. Here we examined influenza virus entry pathways using pharmacological inhibitors and DN mutant proteins in fully polarized MDCK II cells, a well established and robust model for differentiated epithelia [12].

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2. Materials and methods

2.1. Virus preparation

For preparation of virus stocks for infection, approximately 10^3 plaque-forming units (PFU) of influenza virus A/WSN/33 (H1N1) virus were inoculated into 10-day-old specific-pathogen-free chicken embryos. At 48 h post inoculation, allantoic fluid from infected embryos was collected, clarified by centrifugation at 1800×*g* for 15 min at 4 °C, and used as a virus stock. The virus stocks were titered by plaque assay in MDBK (bovine kidney) cells and stored at −80 °C.

2.2. Cell culture

In order to obtain polarized MDCK II epithelial cell culture, MDCK II cells (provided by Dr. Colin Parrish, Cornell University) were grown in DMEM media supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Cellgro) on 0.4 μm semi-permeable Transwell filters (Corning). Polarity was monitored by measurement of the transepithelial electrical resistance (TEER) of the monolayer cultivated on the semi-permeable filter, using an EVOMX meter along with electrodes for cell culture inserts (World Precision Instruments). Before measurement, culture media was changed to fresh warm media for all filter inserts. After being confluent for 3–4 days on Transwell filters, MDCK II reached an average TEER of 230 ohms.cm^2, which was consistent with observations in the literature [13]. MDCK cells (ATCC CCL34) that were not polarized displayed both fibroblast-like and epithelia-like morphology were used as control, due to their inability to form a tight monolayer. The measured TEER of MDCK-CCL34 cells grown under the same culture conditions of MDCK II cells on Transwell filters was 10-fold lower than the TEER of those MDCK II cells.

2.3. Chemical inhibitor treatments and virus infection

Different chemical inhibitors listed below were used to treat fully polarized MDCK II cells and non-polarized MDCK cells for 30 min before infection with influenza virus A/WSN/33 (H1N1), diluted in RPMI 1640 medium containing 0.2% bovine serum albumin (Sigma), 1 mM HEPES, pH 6.8. The inhibitor-treated cells were then incubated with influenza virus at an MOI of 1 (MDCK-CCL34) or an MOI of 5 (MDCK II) for 1 h at 37 °C in the presence of inhibitors. After virus adsorption, the inoculum was replaced with fresh media (DMEM supplemented with 2% fetal bovine serum) and inhibitor for the duration of the incubation period. At 5 h post infection, cells were fixed and analyzed.

2.4. Chemicals and plasmids

Chlorpromazine (CPZ), dynasore, 5-ethylisopropyl amiloride (EIPA), methyl-β-cyclodextrin (MBCD) and nystatin were purchased from Sigma–Aldrich. Dynamin-2 wild type (WT) and DN constructs were obtained from Dr. Mark McNiven of Mayo Clinic Cancer Center. Eps15 WT and DN plasmids were received from Dr. Jennifer Lippincott-Schwartz (NIH). Epsin1 WT and DN plasmids were provided by Dr. Xiaowei Zhuang of Harvard University. Caveolin-1 constructs were a gift from Dr. Ari Helenius (ETH Zürich).

2.5. Immunofluorescence microscopy

Cells were seeded on glass cover slips or Transwell filters. After infection and incubation, cells were fixed with 3% paraformaldehyde (PFA). For detection of influenza infection, we used mouse anti-nucleoprotein (NP) antibody (H16, L10-4R5, ATCC). The secondary antibodies AlexaFluor goat anti-mouse 488 purchased from Molecular Probes. Nuclei were stained with Hoechst 33258 (Molecular Probes). Cover slips with cells were mounted on glass slides using Mowiol. Cells on glass cover slips were examined on a Nikon Eclipse E600 fluorescence microscope equipped with a SensiCam EM camera (Cooke Corp). MDCK II cells on Transwell filters were imaged on a Leica SP5 confocal microscope.

2.6. Western blot

MDCK cells were infected with influenza virus after chemical treatments or mock treatment. Cells were then lysed with RIPA buffer (Millipore) with protease inhibitor cocktail (Roche). The lysate was clarified by centrifugation at 18,000×*g* at 4 °C and mixed with Laemmli buffer with 10% β-mercaptoethanol. Western blot bands were revealed with an antibody against influenza virus matrix (M1) protein (M2-1C6-4R3, ATCC) and anti-mouse secondary antibodies coupled to horseradish peroxidase.

2.7. Electroporation of plasmid DNA

To overcome the difficulty of recombinant protein expression in polarized cells, we used defined electroporation conditions on fully polarized MDCK II cells as described in [14]. 30 μg/ml of plasmid DNA was mixed with electroporation buffer (Eppendorf) and was delivered using an electrode (CUY512-5, Nepagene) connected to an ECM 830 electroporation system (BTX Instruments).

3. Results and discussion

To examine the CME pathway in polarized MDCK cells, we first utilized pharmacological inhibitors that target the CME pathways in host cells. Chlorpromazine (CPZ), which prevents clathrin assembly, has been widely used to block CME including studies on the entry mechanism of influenza viruses in non-polarized cells [1]. Dynasore has an inhibitory effect on GTPase activity of dynamin with low cell toxicity [15]. Since the discovery of dynasore, it has been widely used as specific dynamin inhibitor for entry studies of various virus [16–18]. CPZ or dynasore were used to treat fully polarized MDCK II cells and non-polarized MDCK-CCL34 cells for 30 min before infection with influenza virus A/WSN/33 (H1N1). At 5 h post infection, cells were fixed and analyzed by immunofluorescence microscopy. MDCK-CCL34 cells were more sensitive to the toxicity of CPZ than MDCK II cells; thus lower concentrations of CPZ were used to treat MDCK-CCL34 cells. The quantity of inhibitors used in the treatment did not affect cell viability (data not shown). Treatment with CPZ on both MDCK-CCL34 and MDCK II caused a substantial reduction on the number of NP expressing cells after influenza infection (Fig. 1A) However, dynasore treatment had a more obvious impact on polarized MDCK II cells than their non-polarized counterpart (Fig. 1B). This evidence indicates a potential role of dynamin in the entry pathway of polarized MDCK II cells, while non-polarized MDCK-CCL34 cells do not depend as much on dynamin usage for entry. The effect of CPZ and dynamin on the canine kidney cells were further confirmed by western blot analysis (Fig. 1C).

The pharmacological approaches mentioned above indicate an important role of CME in influenza virus infection in both polarized MDCK II cells and non-polarized MDCK-CCL34 cells. However, we wished to examine specific components of the CME pathway. Conventional transfection methods with cationic lipid or adenovirus transduction are either of low transfection efficiency or not ideal for our study. To overcome the difficulty of recombinant

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