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Enhanced acetylation of alpha-tubulin in influenza A virus infected epithelial cells

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ABSTRACT

Acetylated microtubules (AcMTs), a post-translationally modified form of microtubules, promote polarized protein transport. Here we report that influenza A virus (IAV) induces the acetylation of microtubules in epithelial cells. By employing specific inhibitors and siRNA we demonstrate Rho GTPase-mediated downregulation of tubulin deacetylase activity in IAV-infected cells, resulting in increased tubulin acetylation. Further, we demonstrate that depolymerization/deacetylation or enhanced acetylation of microtubules decreased or increased, respectively, the release of virions from infected cells. IAV assembly requires the polarized delivery of viral components to apical plasma membrane. Our findings suggest the potential involvement of AcMTs in polarized trafficking of IAV components.

1. Introduction

Cytoskeleton is actively involved in virus entry, assembly, and release [1,2]. Cytoskeleton has been proposed to be involved in influenza A virus (IAV) assembly too [3]; however, a precise role of cytoskeletal elements is not understood. IAV causes an acute febrile respiratory disease in humans. IAV, an enveloped virus, is the prototype of family Orthomyxoviridae and possesses a segmented negative-sense RNA genome [4]. It replicates in the nucleus, assembles at the plasma membrane and is released by budding [5]. IAV assembly requires the polarized delivery of viral components to apical plasma membrane. IAV particle consist of three major components: the viral envelope, matrix protein (M1), and viral ribonucleoprotein (vRNP) core. During assembly, constituents of viral envelope are directly transported to the plasma membrane via exocytic pathway. However, transport mechanism of M1 and vRNP to the plasma membrane is not fully understood. The likely possibilities are that they use a piggy-back mechanism on viral transmembrane proteins or cytoskeletal elements [3]. Previously, M1 and nucleoprotein (NP) of IAV were shown to interact with actin filaments [6,7]. Recent reports have described the association of vRNP with microtubules and incorporation of α- and β-tubulin, the heterodimers of microtubules into IAV particles [8–10]. Further, we recently have shown the cleavage of tubulin deacetylase histone deacetylase 6 (HDAC6) by IAV-induced caspase-3 in infected cells [11]. Microtubules-based transport machinery helps many viruses to effect their movement within the cell [2]. Our hypothesis is that IAV modulates the microtubule cytoskeleton to facilitate the transport of viral components. Therefore, we are investigating the modulation of microtubule network and its significance during IAV replication. Here, we show that IAV induces the acetylation of α-tubulin in epithelial cells.

2. Materials and methods

2.1. Cells, virus, and plasmids

Madin-Darby canine kidney (MDCK) and normal human bronchial epithelial (NHBE) (provided by Greg Conner, University of Miami) cells were grown in MEM (Invitrogen) and BEGM (Lonza), respectively. IAV (H1N1) New Caledonia strain was propagated in embryonated chicken eggs and titrated on MDCK cells. Plasmid pcDNA3 (Invitrogen) expressing human HDAC6 was provided by Tso-Pang Yao (Duke University). HDAC6 (D1088E) mutant has been described elsewhere [11].

Abbreviations: AcMTs, acetylated microtubules; AcTub, acetylated α-tubulin; BIM, bisindolylmaleimide; CdTB, *Clostridium difficile* toxin B; HDAC6, histone deacetylase 6; hpi, hour post-infection; IAV, influenza A virus; INF, infected; M1, matrix protein; mDia, mammalian diaphanous protein; MDCK, Madin-Darby canine kidney; MOI, multiplicity of infection; NHBE, normal human bronchial epithelial; Noc, nocodazole; NP, nucleoprotein; PBS, phosphate-buffered saline; TSA, trichostatin A; UNI, uninfected; vRNP, viral ribonucleoprotein

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2.2. Infection and transfection

Cells were washed twice with phosphate-buffered saline (PBS). Virus inoculum, diluted in PBS and supplemented with 3 μg/ml trypsin was added to cells. After 1 h incubation at 37 °C, inoculum was removed and cells were washed once with PBS. Fresh MEM was added and cells were incubated at 37 °C for 24 h. In some experiments, MEM supplemented with bisindolylmaleimide (BIM), *Clostridium difficile* toxin B (CdTB), nocodazole (Noc) (Calbiochem) or trichostatin A (TSA) (Sigma-Aldrich) was added to cells after removing the inoculum and cells were incubated as above. To inactivate IAV, inoculum was exposed to 1–2 mJ of UV radiation. For transfection, 2–3 μg plasmid DNA and 4–5 μl Lipofectamine 2000 (Invitrogen) were diluted separately in OptiMEM I (Invitrogen), mixed and incubated for 20–30 min at room temperature. DNA-Lipofectamine complex was added to cells and cells were incubated for 24 h at 37 °C.

2.3. Western blotting

Cells were harvested, washed with PBS, and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, and protease-inhibitor cocktail [Roche]). The whole cell lysate was resolved on 10% Bis–Tris or 7% Tris-acetate gels (Invitrogen) and proteins were transferred to nitrocellulose membrane. Membrane was probed with mouse anti-acetylated α-tubulin (anti-AcTub), anti-α-tubulin (Sigma-Aldrich), anti-NP (Chemicon), anti-RhoA (Cytoskeleton Inc.) or rabbit anti-actin (Abcam), anti-HDAC6 (Santa Cruz) primary antibodies followed by HRP-conjugated donkey anti-mouse (Affinity BioReagents) or anti-rabbit (Pierce) secondary antibodies, respectively. Protein bands were developed with chemiluminescent kit (Thermo). All the Western blotting steps were performed at room temperature. To re-probe the same membrane with another antibody, membrane was stripped with Restore buffer (Thermo) for 20–30 min at 37 °C.

2.4. siRNA-mediated knockdown

Pre-designed target specific siRNA oligonucleotides against HDAC6 (Catalogue No. sc-35544) and RhoA (Catalogue No. sc-29471) genes were purchased from Santa Cruz Biotech. Oligonucleotides (50 nM) were transfected into cells using 2 μl Lipofectamine RNAiMax (Invitrogen) as described above. After 24–48 h incubation at 37 °C, cells were infected and analyzed by Western blotting as above.

2.5. Real time RT-PCR

Cells were grown on transwell filters (Corning) and infected from apical side. At 24 h post-infection (hpi), apical media was collected and centrifuged at 2000×*g* for 5 min. Viral RNA was then isolated from the media by using QIAamp kit (Qiagen). A quantitative real time RT-PCR assay was performed using viral RNA as a template to amplify IAV M1 gene, in vitro transcribed M1 RNA was used as standard. QuantiTect RT mix (Qiagen), RNA template, M1 forward primer (AGATTGCCGACTCCCAGCATAAGT), reverse primer (TGTTCACTCGATCCAGCCATTTGC), and probe (56-FAM/AGAAC AGAATGGTTCTGGCCAGCACT/3BHQ) were mixed and RT-PCR reaction was run on ABI 7900 thermocycler.

2.6. Virions preparation

Cells were grown on transwell filters and infected from apical side. At 24 hpi, apical media was collected, cleared off cell debris, and layered on 30% sucrose-NTE (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) cushion and centrifuged at 250 000×*g* for 2 h at 4 °C in Beckman TLA55 rotor. The pellet was resuspended in sample buffer and analyzed by Western blotting.

3. Results

3.1. IAV-infected epithelial cells contain elevated levels of AcTub and α-tubulin

We recently demonstrated the cleavage of HDAC6 by IAV-induced caspase-3 in infected cells [11]. This indicated the potential regulation of HDAC6 activity in IAV-infected cells. Because HDAC6 is α-tubulin deacetylase [12], we investigated the acetylation of α-tubulin in IAV-infected cells. MDCK cells were infected with IAV, and total cell lysates were analyzed by Western blotting. We discovered that IAV-infected cells contained elevated level of AcTub (Fig. 1a). Over 2.5-times more AcTub was detected in infected cell lysate as compared to uninfected cell lysate (Fig. 1c). Interestingly, elevated levels of total α-tubulin (Fig. 1a) and β-tubulin (not shown) were also observed at the same time. Over 1.5-fold increase in total α-tubulin level was observed in infected cells (Fig. 1c), suggesting that IAV is potentially regulating the level of total tubulin too. To confirm these observations, levels of AcTub and total α-tubulin were analyzed in cells infected with IAV in the presence of BIM, an inhibitor of IAV replication [13] or with UV-inactivated IAV. No increase was observed in the level of either tubulin in BIM-treated infected cells (Fig. 1a) as well as cells infected with UV-inactivated IAV (Fig. 1b), suggesting that a productive IAV infection is required. Primary NHBE cells, which are the actual target of IAV in vivo, were used to further confirm the observations made in MDCK cells. Likewise, NHBE cells also showed elevated levels of acetylated and total α-tubulin after IAV infection (data not shown).

3.2. Activity of tubulin deacetylase HDAC6 is potentially downregulated in IAV-infected cells

To understand the mechanism of enhanced α-tubulin acetylation, cells were treated with TSA, an HDAC6 inhibitor [12]. As expected, after TSA treatment uninfected cells showed an increase in α-tubulin acetylation due to inhibition of HDAC6's deacetylase activity; however, TSA-induced increase in the acetylation of

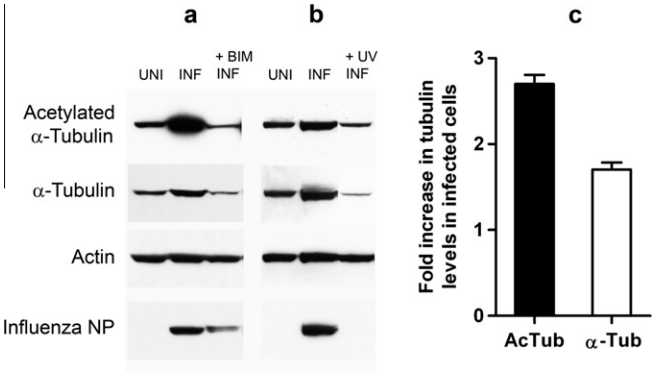

Fig. 1. IAV-infected cells contain elevated levels of AcTub and total α-tubulin. MDCK cells were infected with IAV at MOI of 0.5. Cells were treated with 20 μM BIM (a) or infected with UV-inactivated IAV (b) where indicated. At 24 hpi cells were lysed and total cell lysates were resolved on SDS–PAGE, and indicated proteins were detected by Western blotting. (c) Quantitation of increase in the amount of AcTub and α-tubulin in infected cells. The intensity of AcTub, α-tubulin, and actin bands was quantitated using Quantity One 4.0 software (Bio-Rad). The amount of AcTub and α-tubulin was normalized with actin amount, and was considered a 1-fold increase in uninfected (UNI) cells for comparisons to infected (INF) cells. Each bar represents the mean ± standard deviation (S.D.) for three independent experiments.