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Intravirion cohesion of matrix protein M1 with ribonucleocapsid is a prerequisite of influenza virus infectivity

O.P. Zhirnov [a,*], A.A. Manykin [a], J.S. Rossman [b], H.D. Klenk [c]

[a] D.I. Ivanovsky Institute of Virology, Moscow 123098, Russia
[b] School of Biosciences, University of Kent, Canterbury CT27NJ, UK
[c] Institute of Virology, Philipps University, Marburg 35037, Germany

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ABSTRACT

Influenza virus has two major structural modules, an external lipid envelope and an internal ribonucleocapsid containing the genomic RNA in the form of the ribonucleoprotein (RNP) complex, both of which are interlinked by the matrix protein M1. Here we studied M1-RNP cohesion within virus exposed to acidic pH *in vitro*. The effect of acidification was dependent on the cleavage of the surface glycoprotein HA. Acidic pH caused a loss of intravirion RNP-M1 cohesion and activated RNP polymerase activity in virus with cleaved HA (HA1/2) but not in the uncleaved (HA0) virus. The *in vitro* acidified HA1/2 virus rapidly lost infectivity whereas the HA0 one retained infectivity, following activation by trypsin, suggesting that premature activation and release of the RNP is detrimental to viral infectivity. Rimantadine, an inhibitor of the M2 ion channel, was found to protect the HA1/2 virus interior against acidic disintegration, confirming that M2-dependent proton translocation is essential for the intravirion RNP release and suggesting that the M2 ion channel is only active in virions with cleaved HA. Acidic treatment of both HA0 and HA1/2 influenza viruses induces formation of spikeless bleb-like protrusion of ~25 nm in diameter on the surface of the virion, though only the HA1/2 virus was permeable to protons and permitted RNP release. It is likely that this bleb corresponds to the M2-enriched and M1-depleted focus arising from pinching off of the virus during the completion of budding. Cooperatively, the data suggest that the influenza virus has an asymmetric structure where the M1-mediated organization of the RNP inside the virion is a prerequisite for infectious entry into target cell.

1. Introduction

Influenza virus is a member of the enveloped Orthomyxoviridae family. It contains 2 major structural modules: an internal RNA-containing nucleocapsid surrounded with a lipid envelope. The internal nucleocapsid is a complex of eight segments of viral RNA with the major nucleoprotein NP (~1000 molecules per virion) and the minor polymerase proteins PB1, PA, and PB2. The lipid membrane contains the spike glycoproteins NA and HA and the ion channel protein M2. The channel protein M2 (m.w. 15 kD) is a minor protein (20–60 molecules per virion (Lamb et al., 2001; Zebedee and Lamb, 1988)), which forms homotetramers that when activated by exposure to low pH, transport protons inside the virus particle (Pinto and Lamb, 2006). A network of the major matrix protein M1 (~3000 molecules per virion) locates between nucleocapsid and lipid envelope providing virus integrity by interlinking the nucleocapsid with the lipid envelope.

M1 has been the object of several imaging studies by cryo-electron microscopy, which preserved native virion structure and morphology (Harris et al., 2006; Calder et al., 2010; Fontana and Steven, 2013; Fontana et al., 2012). These studies mainly focused on structural pleomorphism in virus populations, and two principle observations concerning properties of the M1 layer have been made. First, not all particles of influenza A virus grown in chicken eggs were found to contain a clearly resolved dense matrix M1 layer beneath the lipid envelope and the number of such virions lacking the M1 layer markedly increased after short incubation of virus in acidic medium (Fontana et al., 2012; Fontana and Steven, 2013). The authors suggested that the M1 molecules dissociate from each other, as well as from the lipid membrane, during external acidification. Second, using cryo-electron microscopy Harris et al. (2006) observed gaps in the virion M1 layer, which coincides with the absence of overlaying spikes on the outer surface of the virion membrane. The authors speculated that

* Correspondence to: D.I.Ivanovsky Institute of Virology, Gamaleya 16, Moscow 123098, Russia.
E-mail address: zhirnov@inbox.ru (O.P. Zhirnov).

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these gaps may be the remnant of the site of membrane scission formed during virus budding from the host cell.

Earlier, using subvirion fractionation experiments, we found that disintegration of influenza A virions by nonionic detergent in acidic medium solubilized M1 and released the ribonucleoprotein (Zhirnov, 1990; 1992). These experiments suggested that M1-mediated cohesion between nucleocapsids and the lipid envelope was sensitive to acidic pH within intact whole virions. This sensitivity of intravirion interactions to acidic pH could facilitate virion disassembly in acidic endosomes during virus entry and uncoating in the target cell (Helenius, 1992; Lamb and Pinto, 2005). The M2 ion channel conducts protons from the endosomal lumen into virions to dissociate the viral RNPs from the M1 matrix protein. This dissociation can be coordinated with acid-triggered fusion between viral and endosomal membranes to release the RNPs into cytosol (Lamb et al., 2001; Lamb and Pinto, 2005). At the same time, it is reported here, that before M1-RNP dissociation the M1 protein can play a novel active function in organizing an asymmetric localization of RNPs and in translocating RNPs within the virus, promoting its exit into cytoplasm.

To shed light on the virus uncoating machinery we have studied structural and functional alterations in virions exposed to acidic pH. Virus with cleaved HA (HA1+HA2) was found to be permeable to acidic medium, which induced internal disintegration and a loss of infectivity, whereas virions with uncleaved HA (HA0) have a cryptic structural organization that is resistant to acidic pH-mediated disintegration, thus preserving infectivity in the presence of acidic pH. The entry of protons into the virion appeared to proceed through the bleb area formed at a specific single site in the virus envelope. Most probably, this location corresponds to the remnant site of membrane scission formed during virus budding from the host cell plasma membrane and is thus enriched with M2 and depleted with M1 proteins. Taken together, these data suggest that influenza virus possesses an asymmetric internal structural organization, which facilitates the M1-mediated delivery of RNPs from the virion head to the bleb-like virion tail and its subsequent release into the cytosol during virus uncoating. Exposure of intact virions to acidic medium destroys M1-RNP cohesion and intravirion RNP transport, resulting in a loss of virus infectivity.

2. Materials and methods

2.1. Cells and viruses

Laboratory passaged strains of influenza A/Aichi/2/68 and A/Hong Kong/68 (H3N2) (A/HK/68) viruses were propagated in 10-day old embryonated chicken eggs. Freshly isolated A/HK/68 virus was propagated only 4 times in chicken eggs. Virus titers were measured by virus focus assay in Madin Darby canine kidney cells (line MDCK-H) (collection of Institute of Virology, Marburg). MDCK-H cells were passaged in Dulbecco's minimal essential medium (DMEM) containing 10% bovine fetal calf serum (FCS) (GIBCO-BRL, Germany). For infection, 2-day-old confluent MDCK-H cell monolayers were incubated with egg grown influenza virus (about 1 PFU/cell) for 1 h at 37 °C. After infection, cells were washed and incubated with DMEM without serum at 37 °C for 15–17 h and virus-containing culture fluid was then prepared for acidification, microscopic examination, gel electrophoresis, and virus fractionation analysis.

2.2. Virus uncoating centrifugation through glycercol-containing detergent

2.5 ml of Virus-containing culture fluid was clarified at 8500 rpm for 20 min and the supernatant was centrifuged through 2.7 ml of 30% glycerol containing 0.3% of nonionic detergent NP-40, 175 mM NaCl, 20 mM Tris–HCl (pH 7.8) at 30,000 rpm for 3 h at 12 °C (rotor SW 55.1; Beckman centrifuge Optima L-80 XP). Polypeptides in the particulate fraction (containing the nucleocapsid) were solubilized in SDS and electrophoresed in 12% polyacrylamide gels using Tris-glycine-SDS buffer followed by western blot analysis.

2.3. Virus fractionation by differential centrifugation

Samples of culture fluid were centrifuged through a cushion of 25% glycerol prepared in PBS for 2 h at 23,000 rpm in a SW 55.1 rotor (Beckman centrifuge Optima L-80 XP). Virus pellets were suspended in 5 mM phosphate buffer (pH 7.4) containing 70 mM NaCl and 1.35 mM KCl, subdivided into equal parts, treated with 200 μM of Rimantadine hydrochloride (Rim) for 20 min, acidified with 7.5 mM of Sodium acetate buffer (pH 4.5) for 10 and 35 min. Rimantadine (Rim), an inhibitor of the M2 ion channels, was used to study the involvement of these channels in virus interior acidification under virus exposition to acidic pH. Samples were neutralized with 1 M of Tris–HCl (pH 8.0) buffer containing glycerol and detergent NP-40 with the final concentrations 150 mM, 25% and 0.4%, respectively. Mixtures were centrifuged at 24,000 rpm in a SW 55.1 rotor (using 1.5 ml conical tubes MFG 70272; Beckman) for 1.5 h at 11 °C in Spinco L7 ultracentrifuge. Supernatants (soluble protein fraction; Sol) and pellets (nucleocapsid fraction; NC) were prepared and analyzed by SDS-PAGE and WB.

2.4. Infectious focus assay in cultured cells

MDCK-H cells grown in 24-well plates were incubated with 0.3 ml/well of ten-fold virus dilutions in DMEM. After 60-min incubation at 37 °C, the virus inoculum was removed, and cells were washed and incubated with 0.5 ml of DMEM containing 0.5% FCS. 8 h after infection, cells were fixed with 4% paraformaldehyde for 1 h and permeabilized with 0.2% of NP-40 detergent for 2 min. Permeabilized cells were incubated for 1.5 h with anti-influenza virus NP antibodies and then with horseradish HRP-conjugated secondary anti-species antibodies (Dako; Hamburg, Germany), followed by visualization of virus foci with TMB insoluble substrate “True Blue” (KPL) (Zhirnov and Klenk, 2013). Stained virus foci were counted in a microscope at magnification x250.

2.5. Polymerase assay

Virus-containing culture fluid was clarified at 7000 rpm for 20 min and centrifuged through 30% glycerol at 25,000 rpm for 3 h in SW 55.1 rotor (Optima L8-XP centrifuge). Virus pellets were suspended in 50 μl of 20 mM Tris–HCl buffer (pH 8.0) containing 0.4% non-ionic detergent NP-40. 30 μl of virus suspension was mixed with equal volume of 2-fold concentrated polymerase mixture containing 20 mM Tris–HCl (pH 7.8); 250 mM NaCl; 5 mM DTT; 10 mM $MgCl_2$; 2 mM each ATP, GTP, CTP; 0,5 mM ApG; 0.3 mM Biotin-11-UTP (iba-Lifescience; Germany); RNAzin 10 U/ml (Fermentas, Germany) and incubated at 31° C for 90 min. The reaction was terminated by adding 1 μl of 10% SDS, 20 μg of tRNA (Boehringer cat.n.109525, Germany), 120 μl of water and 750 μl of 95% ethanol containing 10 mM of ammonium acetate (pH 5.0) and incubated overnight at 20° C. RNA was pelleted at 14,000 rpm for 20 min and suspended in 30 μl of deionized water containing RNAzin. 10 μl aliquots of each sample were loaded onto one spot of