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Caveolin-1 limits human influenza A virus (H1N1) propagation in mouse embryo-derived fibroblasts

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ABSTRACT

Caveolin expression supports the multiplication of retro-, ortho- and paramyxoviruses in susceptible cells. However, human influenza A virus (IAV), an orthomyxovirus, does not multiply efficiently in mouse embryo fibroblasts (MEFs), which are abundant in caveolin-1 (Cav-1). Surprisingly, the absence of Cav-1 in a MEF cell line removed the block for IAV replication and raised the infectious titer 250-fold, whereas the re-introduction of Cav-1 reversed the effect. The monitoring of cellular pathways revealed that Cav-1 loss considerably increased activities of p53. Furthermore, infection of MEF Cav-1 (−/−) induced reactive oxygen species (ROS) and pronounced apoptosis in the late phase of viral multiplication, but no type I IFN response. Strikingly, pharmacological inactivation showed that the elevated levels of ROS together with apoptosis caused the increase of virus yield. Thus, Cav-1 represents a new negative regulator of IAV infection in MEF that diminishes IAV infectious titer by controlling virus-supportive pathways.

Introduction

Viral growth limitations give valuable insights into pro- and antiviral mechanisms, as they are often caused by the presence or induction of intracellular restriction factors or the absence of cellular dependency factors necessary for efficient virus multiplication. Moreover, the type and density of viral surface receptors and the effectiveness of induced antiviral defense mechanisms are reasons for differences in susceptibility to virus infection (Chin and Brass, 2012; Garcia-Sastre et al., 1998; Koerner et al., 2007; Ma et al., 2011; Seitz et al., 2010; Wang et al., 2007, 2014).

Bronchial/tracheal epithelial cells are the primary target of human influenza A virus (IAV) infection. Compared with the main target cells, other cell types exhibit lower permissiveness for influenza virus replication. For example, adjacent immune cells such as macrophages can be productively infected, albeit to a lower extent (Manicassamy et al., 2010). Moreover, virus restriction has been found in human mast cells, from which fewer viruses are released compared with other permissive cell lines (Marcet et al., 2012). Furthermore, mouse fibroblasts only weakly support the replication of human influenza viruses and it has been suggested that viral replication is aborted at a nuclear stage in infected cells (Garcia-Sastre et al., 1998).

Influenza virus multiplication depends on components of the host machinery. For efficient infection and dissemination in tissue, the influenza hemagglutinin protein (HA) must be cleaved into its subunits by cellular proteases (Böttcher-Friebertshäuser et al., 2012; Böttcher et al., 2006; Garten and Klenk, 2008). IAVs enter the cell via receptor-mediated endocytosis and a second non-caveolar entry mechanism (Lakadamyali et al., 2004). The entry is dependent on the type of the receptor determinant: human influenza viruses prefer binding to α2,6-linked neuraminic acid residues present on glycoproteins and glycolipids, whereas avian influenza viruses use α-2,3-conjugated neuraminic acids for entry. A V-type ATPase, a proton-transporting enzyme that functions in acidification and fusion, has been identified as a host factor in genome-wide screening based on RNA interference (Chin and Brass, 2012; Watanabe et al., 2010). Influenza particles enter the nucleus in a manner dependent on members of the importin

Abbreviations: A549, adenocarcinomic human alveolar basal epithelial cells; Cav-1, caveolin-1; CBM, caveolin-1 binding motif; CMV-IE, cytomegalovirus immediate early promoter enhancer; ESCRT, Endosomal sorting complexes required for transport; HA, hemagglutinin; IAV, influenza A virus; M2, matrix protein 2; MDCK, Madin–Darby canine kidney cells; MEF, mouse embryo fibroblast; NAC, N-acetyl cysteine; NRF2, nuclear erythroid p45 related factor 2; NEP, nuclear export protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B-cells; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; ROS, reactive oxygen species; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; vRNP, viral ribonucleoprotein complex

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family (Gabriel et al., 2008, 2011). Nuclear export is also dependent on active and passive mechanisms to aid the timely export of newly synthesized vRNP (viral ribonucleoprotein) to the plasma membrane. In particular, the active nuclear export of vRNPs is triggered by HA accumulation at the plasma membrane followed by activation of the MEK (also known as mitogen-activated protein kinase kinase)/extracellular signal-regulated kinase (ERK) pathway (Marjuki et al., 2006). The concerted apoptosis in the late phase of viral replication then increases the accumulation of vRNP in the cytoplasm through nuclear pore widening by caspase 3 action (Wurzer et al., 2003, 2004). This is remarkable, since, in the past, both apoptosis and formation of reactive oxygen species (ROS) have been considered to limit replication and the spread of viruses. Lately, however, virus-supportive effects have been increasingly observed for both mechanisms (Bottero et al., 2013; Herold et al., 2012; Wurzer et al., 2003). Apart from the contribution of actin and Rab11 to filamentous virus budding (Bruce et al., 2010) and the involvement of RACK1 (receptor for activated C kinase 1), few other determinants are needed for the assembly and release of IAVs. Influenza viruses require the crosstalk of cellular RACK1 protein with its M1 protein for release (Demirov et al., 2012), and they apparently do not rely on ESCRT-mediated exit (endosomal sorting complexes required for transport) (Bruce et al., 2009; Chen and Lamb, 2008; Goettlinger, 2010; Rossman et al., 2010; Rossman and Lamb, 2011). Strikingly, filamentous influenza virus pinching-off is dependent on the M2 protein, which solely makes use of a short amphipathic helix in M2 to induce curvature and subsequent scission of the budding virus (Rossman et al., 2010).

In our investigation, we have analyzed the role of Cav-1 in human IAV infection of mouse fibroblasts. Cav-1 is a lipid-raft-resident protein involved in caveolae formation, cellular signaling, and cholesterol transport and its homeostasis (Liu et al., 2002). In general, Cav-1 modulates virus propagation by interaction with various viral proteins (Brown et al., 2002; Hovanessian et al., 2004; Huang et al., 2007; Mir et al., 2007; Parr et al., 2006; Ravid et al., 2010; Sun et al., 2010; Yu et al., 2006; Zou et al., 2009). Several binding regions have been described (Ball et al., 2013; Hovanessian et al., 2004; Huang et al., 2007; Ravid et al., 2010; Sun et al., 2010). Intriguingly, Ravid et al. (2010) have demonstrated that Cav-1 binds to the viral M protein of parainfluenza virus (PIV5), a paramyxovirus, through a defined caveolin-binding motif (CBM), and that the protein is important for assembly and budding. Recently, Cav-1 has also been shown to be able positively to modulate the propagation of IAV in Madin–Darby canine kidney (MDCK) cells (Sun et al., 2010), presumably by binding to M2 (Sun et al., 2010; Zou et al., 2009). In addition to the structural support of Cav-1 in the transport and localization of foreign proteins to lipid rafts, its involvement in signaling has gained much interest over the last few years (for reviews, see Boscher and Nabi (2012) and Liu et al. (2002)). Thereby, Cav-1 serves as a scaffold to organize signaling-competent complexes in lipid rafts and caveolae (Liu et al., 2002).

Here, we report that the Cav-1 depletion in mouse fibroblasts results in a 250-fold increase in the yield of infectious human influenza A (H1N1) virus. Reporter gene arrays and biochemical assays have revealed that the infection of Cav-1-depleted mouse embryo fibroblast (MEF) cells with IAV PR/8/34 severely induces stress/toxicity response mechanisms. Results from inhibition experiments have shown that the timely induction of apoptosis and the appearance of ROS particularly cause a considerable increase in the production of infectious virus. Furthermore, whereas a low activity of transcription factors responsible for the expression of type-I-interferon-stimulated genes is observed upon H1N1 infection of MEF wildtypes (MEF WT), such a response is absent in PR8-infected MEF Cav-1 (−/−) cells. Thus, the stress/toxicity response and, presumably, the impairment of components of the innate immune response contribute to increased virus production.

Results

MEFs yield increased infectious titer in the absence of Cav-1

Mouse fibroblasts exhibit a low permissiveness for the propagation of human IAV (Garcia-Sastre et al., 1998). Usually, infectious titers have been reported that are 3–4 orders of magnitude lower than titers from permissive cell lines such as MDCK (Garcia-Sastre et al., 1998). To investigate the influence of Cav-1, which supports retrovirus, paramyxovirus, and orthomyxovirus replication in permissive cell lines (Hovanessian et al., 2004; Huang et al., 2007; Ravid et al., 2010; Sun et al., 2010; Yu et al., 2006; Zou et al., 2009), we investigated the effect of Cav-1 loss on human influenza virus production in a Cav-1-deficient MEF fibroblast line (3T3 MEF KO also refered to as MEF Cav-1 (−/−), ATCC CRL 2753) derived from a Cav-1 knock-out mouse (Razani and Lisanti, 2001). For this purpose 3T3 MEF KO were infected with A/PR/8/1934 at a multiplicity of infection (MOI) of 0.5 for 1 h, and infectious virus titers were determined 24 h post-infection by plaque assay on MDCK and compared with the titers achieved by the parental cell line (3T3 MEF WT, ATCC CRL 2572) (Fig. 1). Intriguingly, a 250-fold increase could be observed, on average, in the specific productivity of infectious virus in the absence of Cav-1.

To exclude that selected cell populations arising during the establishment of the Cav-1 KO cell line could contribute to the increase in virus production, we performed Cav-1 knock-down experiments with IAV infected 3T3 MEF WT cells (Fig. 2A). For this purpose, retroviral transduction was used to introduce a Cav-1 shRNAi into MEF WT and to select for integration events yielding MEF KD Cav-1 as described earlier (Schuck et al., 2004; Sun et al., 2010). At 24 h after infection, IAV production from 3T3 MEF WT, MEF KD Cav-1, and MEF transduced with the empty retroviral vector was compared. On average, MEF KD Cav-1 cells yielded 2.7-fold more infectious virus than 3T3 MEF cells. The transduction of

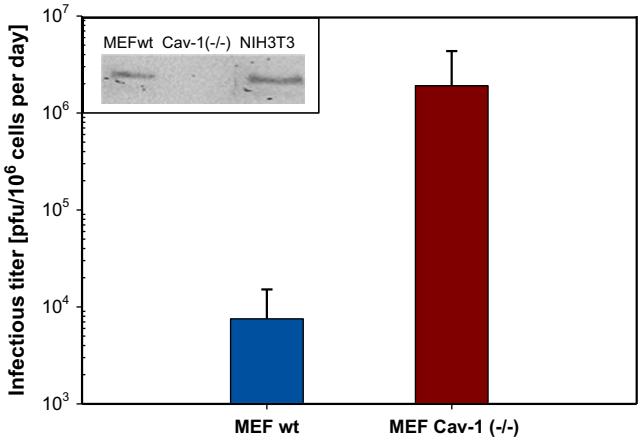

Fig. 1. Effect of caveolin-1 depletion on human influenza A virus (H1N1) production in mouse fibroblasts. Mouse embryo fibroblasts (MEF WT) and Cav-1 (−/−) deficient MEFs were infected with an MOI of 0.5 with influenza A virus (PR/8/1934) for 1 h and incubated in production medium for 24 h. The titer of infectious virus was determined from filtered supernatants on MDCK cells by using a plaque assay. Virus titers are given in plaque-forming units (pfu) per one million cells in a production period of 1 day. Statistical analysis was performed by using the paired *t*-test ($n=6$, $^*p<0.05$). Inset: Western blot detection of Cav-1. The 24 kD signal (α-form) is abundant in cell extracts from mouse embryo fibroblasts 3T3 MEF WT and NIH3T3, but is absent in MEF Cav-1 (−/−).