Virology 525 (2018) 161–169

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

The avian influenza virus PA segment mediates strain-specific antagonism of BST-2/tetherin

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ARTICLE INFO

Keywords:
BST-2
Tetherin
Influenza virus
Avian influenza
PA
PA-X
NS1

ABSTRACT

BST-2 is an antiviral protein described as a powerful cross-species transmission barrier for simian immunodeficiency viruses. Influenza viruses appear to interact with BST-2, raising the possibility that BST-2 may be a barrier for cross-species transmission. An MDCK-based cell line expressing human BST-2 was generated to study human-derived A/Puerto Rico/8/36 (H1N1; PR8) as well as two low pathogenic avian influenza viruses (subtypes H4N6 and H6N1). The H4N6 and H6N1 viruses were less affected by BST-2 expression than PR8, due to their ability to decrease BST-2 levels, a function localized to the PA segment of both avian viruses. Experiments with PA-mutant and -chimeric viruses confirmed that the avian PA segment conferred BST-2 downregulation and antagonism. These results indicate a species-specific ability of PA from low pathogenic avian viruses to mitigate human BST-2 antiviral activity, suggesting that BST-2 is unlikely to be a general cross-species barrier to transmission of such viruses to humans.

1. Introduction

BST-2 (bone marrow stromal cell antigen 2), also known as tetherin, CD317 or HM1.24, is an interferon (IFN)-induced cellular protein that was initially described in 2008 as a restriction factor for human immunodeficiency virus type 1 (HIV-1) (Neil et al., 2008; Van Damme et al., 2008). While BST-2 has been almost exclusively studied as a mammalian antiviral protein, an *in silico* study identified a BST-2 ortholog as far back in vertebrate evolution as the elephant shark, dating the appearance of this gene to over 450 million years ago (Heusinger et al., 2015). Other than in fish, this study also identified orthologs in marsupials, reptiles, and birds, with alligator BST-2 being tested and found to possess antiviral function against HIV-1 release. Among birds, BST-2 was found in turkeys and chickens, but appears to have been lost through gene erosion among many bird species.

As a type II membrane protein, BST-2 possesses a C-terminal GPI (glycophosphatidylinositol) modification and an N-terminal transmembrane domain flanking an extracellular coiled coil central region, and is present on the cell surface as a homodimer (Kupzig et al., 2003). The resulting four-membrane-anchor conformation is considered central to the ability of BST-2 to restrict HIV-1 virion release, where it acts as a "tether" linking the membranes of budded virions to the host cell membrane (Perez-Caballero et al., 2009). As BST-2 acts directly upon the host cell membrane rather than viral components, BST-2 does not target a specific virus but rather has been found capable of restricting virion release and spread for a range of enveloped viruses beyond the retroviruses, such as filoviruses (Jouvenet et al., 2009; Kaletsky et al., 2009; Sakuma et al., 2009), arenaviruses (Radoshitzky et al., 2010; Sakuma et al., 2009), and various coronaviruses (Taylor et al., 2015; Wang et al., 2014).

Many of the viruses described to be sensitive to BST-2 restriction are zoonotic. Most notably, BST-2 has been described as a cross-species transmission barrier that shaped the evolution of the simian immunodeficiency virus (SIV) and HIV (Evans et al., 2010). A recent publication also demonstrated the possible role of BST-2 as a cross-species transmission barrier for various orthobunyaviruses (Varela 2017), with human viruses being restricted by sheep BST-2 but not the human ortholog, and vice versa. Also, equine BST-2 was observed to restrict the growth of both equine and human influenza viruses more effectively than human BST-2 (Wang et al., 2018).

Early reports examining the interplay between influenza viruses and BST-2 suggested that virus-like particles (VLPs) but not wild-type viruses were susceptible to human BST-2 restriction (Watanabe et al., 2011, Bruce et al., 2012). These observations lent credence to the possibility that influenza viruses universally encode an antagonist to BST-2. Contradictory reports soon emerged, however, of viruses inherently sensitive to BST-2 restriction (Gnirss et al., 2015; Hu et al., 2017; Mangeat et al., 2012). Differential abilities of various influenza

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https://doi.org/10.1016/j.virol.2018.09.016
Received 16 August 2018; Received in revised form 19 September 2018; Accepted 21 September 2018

virus neuraminidases (NA) in circumventing BST-2 activity (Leyva-Grado et al., 2014; Yondola et al., 2011) made it apparent that influenza virus sensitivity to BST-2 is likely to be strain-specific. Further studies supported the possibility that influenza virus NA acts a strain-specific antagonist to BST-2 (Leyva-Grado et al., 2014; Mangeat et al., 2012). Mangeat et al. also reported a reduction in BST-2 protein expression, which may be associated with hemagglutinin (HA) and NA together (Gnirss et al., 2015) or the M2 protein (Hu et al., 2017), but their observations of decreased BST-2 mRNA levels during influenza virus infection remained unexplained. Given the variety of influenza strains, host cells, and methodologies used to study the BST-2–influenza interplay, the conflicting data, at the very least, appear to suggest that a variety of influenza virus strains interact with and counteract BST-2 in some fashion.

Given that influenza is and remains an important zoonotic disease, the possibility of BST-2 being a host restriction factor that acts as a cross-species transmission barrier for avian influenza viruses is intriguing. Thus far, however, studies examining the intersection between BST-2 and influenza viruses have mostly focused on human viruses, whether laboratory-adapted, seasonal, or pandemic. These viruses have been successful in maintaining themselves in the human population, suggesting that they possess an inherent capacity to circumvent or antagonize the antiviral activity of BST-2. Therefore, we were interested in comparing human and low pathogenic avian influenza virus strains, which are generally not deemed a direct threat to human health, with the goal of identifying differences in their response to BST-2.

2. Results

2.1. Generation of BST-2-expressing cell lines

To study the impact of BST-2 on human and avian influenza viruses, we first generated an MDCK cell line stably expressing human BST-2 cloned from HeLa cells (Narkpuk et al., 2014) (Supplementary Fig. 1). The MDCK-BST-2 cell line generated by lentiviral transduction was examined by immunofluorescence staining and BST-2 was observed as punctate clusters on cell surface membranes (Fig. 1A), in accordance with expected BST-2 localization. Cells were also probed for BST-2 expression by western blotting and revealed the characteristic pattern of monomeric and dimeric BST-2 in its various glycosylated forms (Fig. 1B).

2.2. Low pathogenic avian viruses are resistant to human BST-2

To test for strain-specific differences in sensitivity to human BST-2, we compared the low pathogenic avian viruses A/duck/Hong Kong/365/78 (H4N6) and A/duck/Suphanburi/AI157/2005 (H6N1) against A/Puerto Rico/8/34 (H1N1) (PR8 for short). These viruses were used to infect MDCK and MDCK-BST-2 cells at a low multiplicity of infection (MOI), and their supernatants were harvested at 48 h post-infection for plaque titration on MDCK cells. As low pathogenic avian viruses have not been reported to cause disease in humans, we expected that the H4N6 and H6N1 viruses would be more susceptible to human BST-2 antiviral activity than PR8. Surprisingly, the PR8 virus exhibited greater sensitivity to BST-2, with titers decreasing by around 1 log in MDCK-BST-2 cells (Fig. 2A). Neither the H4N6 nor H6N1 virus appeared to be negatively affected by the presence of BST-2, with H4N6 showing a slight trend towards increased replication in MDCK-BST-2 cells (Fig. 2B, C). When infected cells were probed to confirm BST-2 expression, western blotting revealed a dramatic disappearance of BST-2 protein in MDCK-BST-2 cells infected by H4N6 and H6N1 viruses (Fig. 2D). Similar levels of BST-2 protein down-regulation were not observed with PR8 infection prior to complete cell death. Analysis of BST-2 mRNA demonstrated that this reduction was accompanied by reduced mRNA levels as well (Fig. 2E). PR8 infection also resulted in decreased BST-2 mRNA levels, albeit not as drastically as infection with the avian viruses. While this result reflects the ability of influenza A viruses in general to mediate host shut-off (Rivas et al., 2016), it also suggests a difference in mRNA down-regulation that may be associated with decreased levels of BST-2. Alternatively, it is also possible that mRNA and protein down-regulation effects occur through distinct mechanisms.

These avian viruses should not have been under selection pressure to develop a specific antagonistic mechanism against human BST-2, due to the nature of their host range. These observations therefore pointed to a general host response inhibition factor present in the virus. The influenza virus nonstructural protein 1 (NS1), in particular, is known for its ability to inhibit an IFN-induced antiviral state through a variety of activities such as blocking host sensors of viral RNA and signal transduction pathways leading to induction of IFN expression (Marc, 2014). As BST-2 expression in our cell line is driven by a constitutive promoter, however, we do not expect that these particular activities will be involved in BST-2 down-regulation.

2.3. The PA segment encodes a common BST-2 antagonist in avian influenza viruses

To determine which viral factors of H4N6 and H6N1 are involved in decreasing BST-2 levels, we studied the impact of each of their genomic segments on BST-2 expression in the context of transfection. We amplified each segment from viral RNA for cloning into the reverse genetics vector pHW2000. Individual genomic segments of PR8, H4N6, and H6N1 were then co-transfected with a BST-2 expression plasmid into HEK293T cells for western blot analysis. While none of the PR8 genomic segments affected BST-2 expression (Fig. 3A), the PA of H4N6 and H6N1 notably reduced BST-2 protein levels (Fig. 3B, C). Additionally, the H6N1 NS segment also exhibited the capacity to reduce protein expression, which is likely due to NS1-associated host shut-off capabilities (Marc, 2014).

As the effect of the PA segment appeared common to both low pathogenic avian viruses, we further tested the effect of PA on BST-2

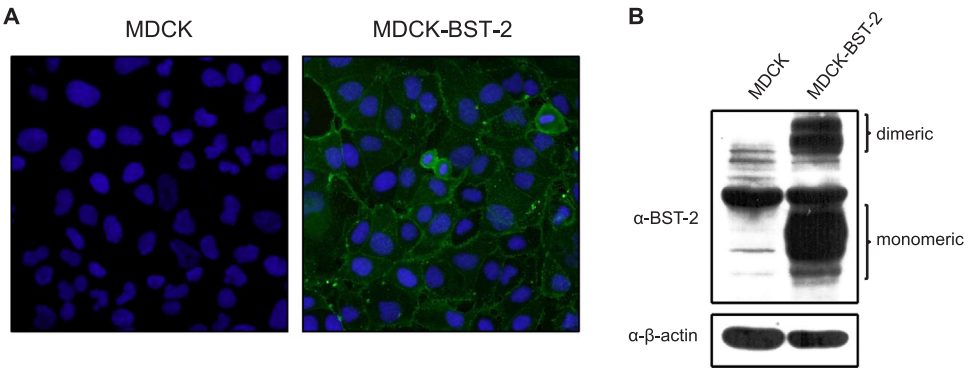

Fig. 1. Expression of the human BST-2 transgene in MDCK cells. (A) MDCK and MDCK-BST-2 cells were fixed with formaldehyde and probed for BST-2 expression (green). Cells were stained with DAPI prior to imaging by fluorescence microscopy. (B) MDCK and MDCK-BST-2 cells were plated in 6-well plates and subsequently harvested. Cell lysates were analyzed by western blotting for BST-2 and β-actin expression.