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An unconventional BST-2 function: Down-regulation of transient protein expression

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ABSTRACT

While viral inhibition by tethering of budding virions to host cell membranes has been focused upon as one of the main functions of BST-2/tetherin, BST-2 is thought to possess other functions as well. Overexpression of BST-2 was found here to down-regulate transient protein expression. Removal of the N- and C-terminal regions of BST-2, previously described to be involved in signal transduction, reduced the impact of BST-2. These results suggest that BST-2-mediated signaling may play a role in regulating the levels of transiently expressed proteins, highlighting a new function for BST-2 that may also have implications for viral inhibition.

1. Introduction

BST-2, also known as CD317, HM1.24 or tetherin, was originally identified as a marker of terminally differentiated B cells [1], and subsequently found to be expressed by most cell types upon interferon stimulation [2]. It is a type II integral membrane protein with an N-terminal transmembrane domain and a C-terminal GPI anchor [3]; its ectodomain forms a coiled-coil with three conserved cysteine residues forming intermolecular disulfide bridges to generate homodimers [4,5].

BST-2 was first reported to possess antiviral function when it was observed to "tether" HIV-1 virions to the host cell membrane [6,7]. This mode of action is dependent upon BST-2 localization to membrane regions associated with the budding of enveloped viruses by virtue of its membrane-bound N- and C-terminal domains. Because no specific recognition of any viral component is required for such inhibition, BST-2-mediated inhibition of viral release has accordingly been demonstrated against a myriad of enveloped viruses, including retroviruses [6,7], filoviruses [8–10], rhabdoviruses [11,12], and alphaviruses [13].

The effect of BST-2 on influenza viruses has also been studied. Human BST-2 does not appear to affect replication of infectious viruses [14–16], but has been reported to inhibit the release of virus-like particles [14,17]. Our own work with influenza viruses, however, revealed great sensitivity of the A/Puerto Rico/8/34 virus (PR8) to BST-2 similar to that reported by Mangeat et al. [18]. Interestingly, the greater sensitivity was observed during the reverse genetics process, which involves initial generation of viral proteins by *de novo* transcription and translation from plasmids encoding viral sequences followed by amplification by multiple rounds of replication, when compared to viral infection. This apparent discrepancy raised the possibility that the observed effects of BST-2 were occurring via different pathways. Indeed, multiple functions have been ascribed to BST-2, including interaction with the cytoskeleton and organization of membrane microdomains [3,19–21] as well as viral sensing and intracellular signaling [22–25]. Our investigation into how BST-2 may have inhibited influenza virus reverse genetics led us to the demonstration that BST-2 is capable of down-regulating transient protein expression and identification of a potential novel BST-2 antiviral mechanism.

2. Materials and methods

2.1. Cells and plasmids

The human embryonic kidney cell line HEK293T and the Madin-Darby canine kidney cell line MDCK were maintained in Opti-MEM (Life Technologies) supplemented with 10% fetal bovine serum. The pHW2000-based reverse genetics plasmids for A/Puerto Rico/

Abbreviations: PR8, A/Puerto Rico/8/34; HAU, hemagglutination units; NP, nucleoprotein.

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8/34 (H1N1) (PR8 for short) were a gift from Dr. Robert Webster (St. Jude Children's Research Hospital, USA) [26]. The pVpu plasmid was a gift from Dr. Klaus Strebel (National Institute of Allergy and Infectious Diseases, USA). Human BST-2 was amplified by RT-PCR from HeLa RNA extracts and cloned into the pHW2000 vector to generate pHW-BST-2. The polymerase I promoter in pHW-BST-2 was removed to generate pBST-2, where BST-2 transcription is driven by a CMV promoter. The truncated ΔBST-2 mutant was generated by mutating the methionine at residue 12 to a stop codon. Other mutant BST-2 constructs were generated by site-directed mutagenesis based on the mutants reported previously by Perez-Caballero et al. [5]. The Igκ secretory leader peptide was amplified from pSecTag (Invitrogen) and fused to a BST-2 mutant lacking the N-terminal transmembrane domain to generate the ΔNt BST-2 mutant. To generate the human EF1α promoter-driven luciferase expression plasmid, the human EF1α promoter was amplified from pBudCE4.1 (Invitrogen) and inserted into the promoterless pGL3E plasmid (Promega) to generate pEF1a-Luc.

2.2. Reverse genetics of PR8

HEK293T and MDCK cells were co-cultured in serum-free Opti-MEM in 6-well plates. Eight pHW2000-based plasmids encoding the 8 genomic segments of the PR8 virus were transfected with or without pHW-BST-2, pVpu, anti-BST-2 siRNA (Santa Cruz Biotechnology) or ΔBST-2. After 6 h, the transfection media was removed and replaced with fresh serum-free Opti-MEM. Twenty-four hours post-transfection, the medium was supplemented with 1 μg/mL TPCK-treated trypsin. Supernatants were harvested at various time points after transfection and the presence of viral particles measured by hemagglutination assay.

2.3. Western blotting

HEK293T cells were plated in 6-well plates and transfected with pHW-NP (an expression vector expressing the nucleoprotein, or NP, of PR8) with or without wild-type or mutant BST-2 and shRNA. The anti-BST-2 shRNA and control shRNA were purchased from Santa Cruz Biotechnology. Five hundred nanograms of each plasmid were used, unless otherwise indicated. Transfected cells were harvested after 72 h and lysed in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1% ProteoBlock Protease Inhibitor Cocktail (ThermoScientific). Lysates were loaded onto 12% polyacrylamide gels for electrophoresis and the separated proteins transferred onto nitrocellulose membranes. The membranes were probed with various antibodies for protein detection: a rabbit anti-human BST-2 antibody (Santa Cruz Biotechnology) followed by a horse-radish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology) to detect BST-2; a mouse anti-influenza A NP antibody (Southern Biotech) followed by an HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology) to detect viral NP; rabbit anti-Vpu serum (anti-Vpu-C; NIH AIDS Reagent Program, NIAID, NIH deposited by Drs. Beatrice H. Hahn and Matthias H. Kraus [27]) was used to detect Vpu; a goat anti-H1N1 antibody (ViroStat) followed by a rabbit anti-goat antibody (KPL) to detect M1; and a mouse anti-β-actin antibody (Santa Cruz Biotechnology) followed by an HRP-conjugated goat anti-mouse antibody to detect β-actin. Bands were visualized using the Clarity Western ECL Substrate (Bio-Rad).

2.4. Luciferase expression

HEK293T cells were plated in 96-well white, clear-bottomed plates and transfected with 100 ng pEF1α-Luc along with varying amounts of pBST-2. When included, 100 ng of anti-BST-2 or control shRNA were used. After 48 h, luciferase expression was assessed by addition of ONE-Glo luciferase substrate (Promega). Luminescence was measured by spectrophotometry.

2.5. mRNA quantitation

Total RNA from transfected cells was extracted and further treated with DNase I. The mRNA of NP, BST-2 and fibronectin were amplified by quantitative real-time RT-PCR using specific primers (primer sequences available upon request) using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad). The ΔCt values for NP and BST-2 were generated against fibronectin and fold expression calculated using the formula $2^{-\Delta Ct}$. Results are illustrated for fold expression relative to NP in the absence of BST-2.

3. Results

Previous reports concerning the impact of BST-2 on influenza virus replication have demonstrated that BST-2 has little to no effect on infectious influenza virus. This stands in seeming contrast to a report by Mangeat et al. [18] and our own observations that human BST-2 significantly decreases virion yield by reverse genetics (Fig. 1). In the presence of BST-2, hemagglutination titers were extremely low even after 60 h, reflecting decreased output of viral particles, without distinguishing between infectious and non-infectious particles (Fig. 1A). This decrease in hemagglutination titers was dose-dependent, with increasing BST-2 input resulting in lower titers (Fig. 1B). Furthermore, this activity was specifically mediated by BST-2, as demonstrated both the ability of anti-human BST-2 siRNA to restore viral particle levels in the supernatant and the inability of the ΔBST-2 mutant to decrease hemagglutination titers. Interestingly, Vpu, an HIV-1 accessory protein known to act as an antagonist against BST-2-mediated inhibition of HIV-1 release [6,7], failed to rescue viral production, suggesting that BST-2 inhibition of influenza virus reverse genetics may occur via a pathway distinct from its inhibition of HIV-1 particle release. Western blot controls reflect the dose-dependent expression of monomeric and dimeric BST-2 and their various glycosylated forms, as well as decreased BST-2 levels in the presence of HIV-1 Vpu and anti-BST-2 siRNA (Fig. 1C). Vpu did not completely abrogate BST-2 expression, in agreement with previous reports that Vpu antagonizes BST-2 only in part by targeting cell surface BST-2 for degradation [28,29]. A major mechanism for BST-2 antagonism by Vpu appears to occur via sequestration of BST-2 in the ER prior to BST-2 trafficking to the surface membrane [30–32].

Viral protein levels were also assessed by examination of NP and the matrix (M1) protein by Western blotting, which revealed decreased expression corresponding to the decrease in viral output (Fig. 1D). The decrease in M1 levels stands in striking contrast to the report by Mangeat et al. [18], where no difference was seen and other aspects of BST-2-influenza virus interaction were subsequently investigated. This discordance may have arisen from differences in the amount of BST-2 plasmid used or their higher TPCK-trypsin concentration in the cell culture media, which may have resulted in some digestion of cell surface BST-2.

The major difference in virus generation between influenza virus reverse genetics and infection is that the former requires *de novo* synthesis of the NP and polymerase complex proteins from expression plasmids by the transfected host cell, while in the latter case, infectious viruses come packaged with these proteins which can immediately initiate viral replication. Therefore, we examined whether BST-2 overexpression affected this critical initial stage of reverse genetics by assessing its impact on NP protein levels. Plasmids pBST-2 and pHW-NP were co-transfected into HEK293T cells, and Western blotting of cell lysates revealed that NP protein levels

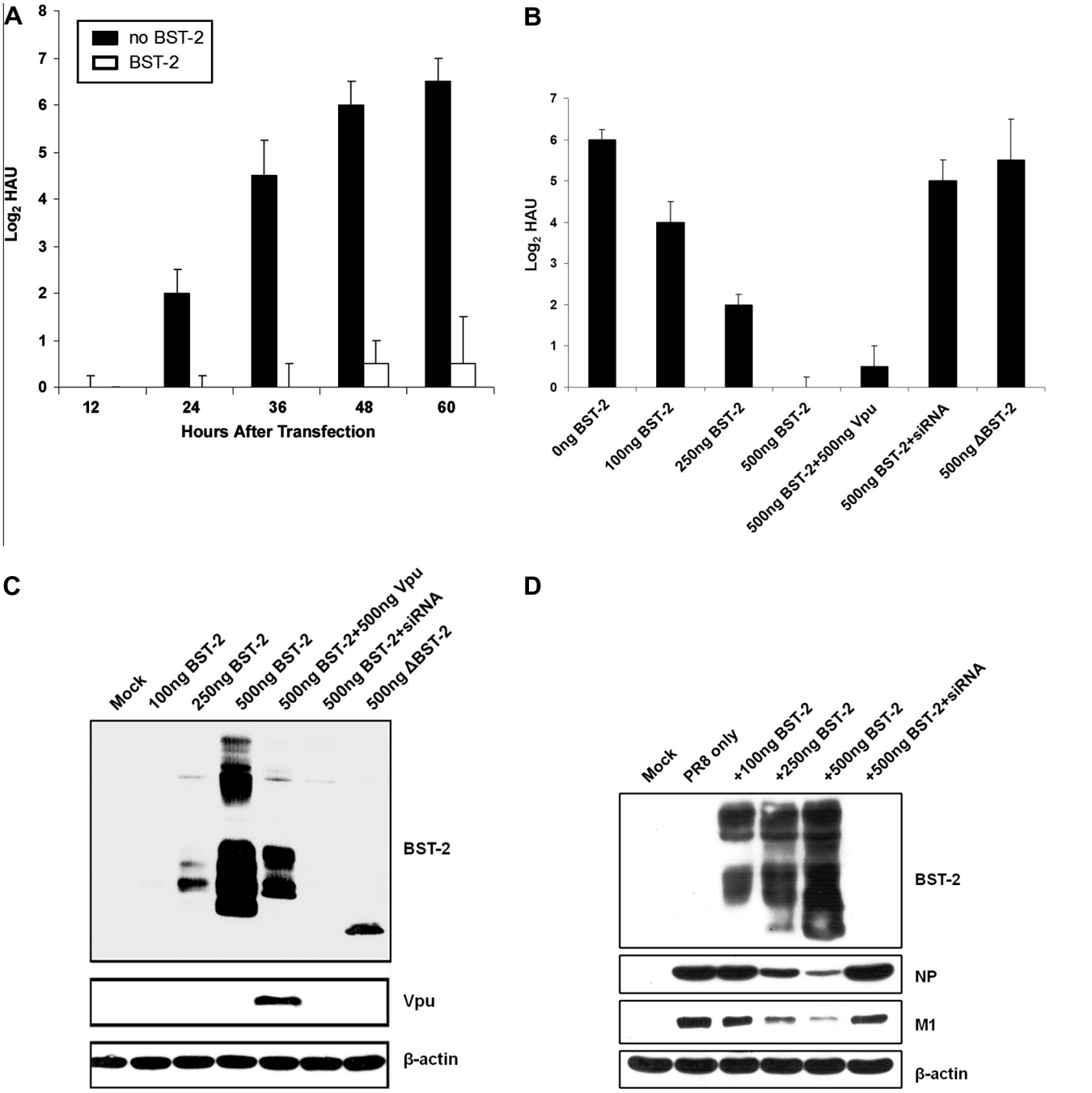

Fig. 1. BST-2 decreases viral particle output and NP levels. (A) HEK293T-MDCK co-cultures were transfected with plasmids encoding the eight genes of PR8 with or without BST-2. At various time points post-transfection, supernatants were sampled and tested for the presence of virus particles by hemagglutination assay. (B) Transfection of HEK293T-MDCK co-cultures was performed as described in (A) in the presence of siRNA against BST-2 or plasmid expressing codon-optimized Vpu. At 48 h post-transfection, supernatants were sampled and tested for the presence of virus particles by hemagglutination assay. (C) HEK293T cells were plated in 6-well plates and transfected with the indicated plasmids. After 72 h, cell lysates were harvested and analyzed by Western blotting. (D) Transfection of HEK293T-MDCK co-cultures was performed as described in (A). After 72 h, cell lysates were harvested and analyzed by Western blotting. Error bars indicate SD. HAU, hemagglutination units.

were indeed negatively affected in a specific and dose-dependent manner by BST-2 (Fig. 2A). As BST-2 has been reported to activate the NF-κB transcription factor [24,33,34], we examined RNA levels to determine whether the impact on NP expression occurred at the level of transcription. Total RNA was extracted from HEK293T cells transfected with NP and BST-2 expression plasmids, and NP, BST-2 and fibronectin mRNA levels were measured by quantitative real-time RT-PCR. No significant impact on NP mRNA levels appeared to be correlated with BST-2 levels (Fig. 2B), suggesting that the previously observed impact of BST-2 on NP protein levels may have occurred post-transcriptionally.

As both the N-terminal transmembrane domain and C-terminal GPI anchor of BST-2 have been reported to play roles in signaling [23,24,33,34], we tested the possibility that these domains may be important for the observed decrease in transient protein expression during BST-2 overexpression. We generated BST-2 mutants lacking either the N- or C-terminus, and then co-transfected their expression plasmids along with pHW-NP. BST-2 and NP protein levels were examined by Western blotting (Fig. 3). Loss of either the N- or C-terminus was observed to reduce the impact of the mutant BST-2 on NP protein levels. In particular, loss of the GPI anchor by deletion of the C-terminus resulted in noticeably

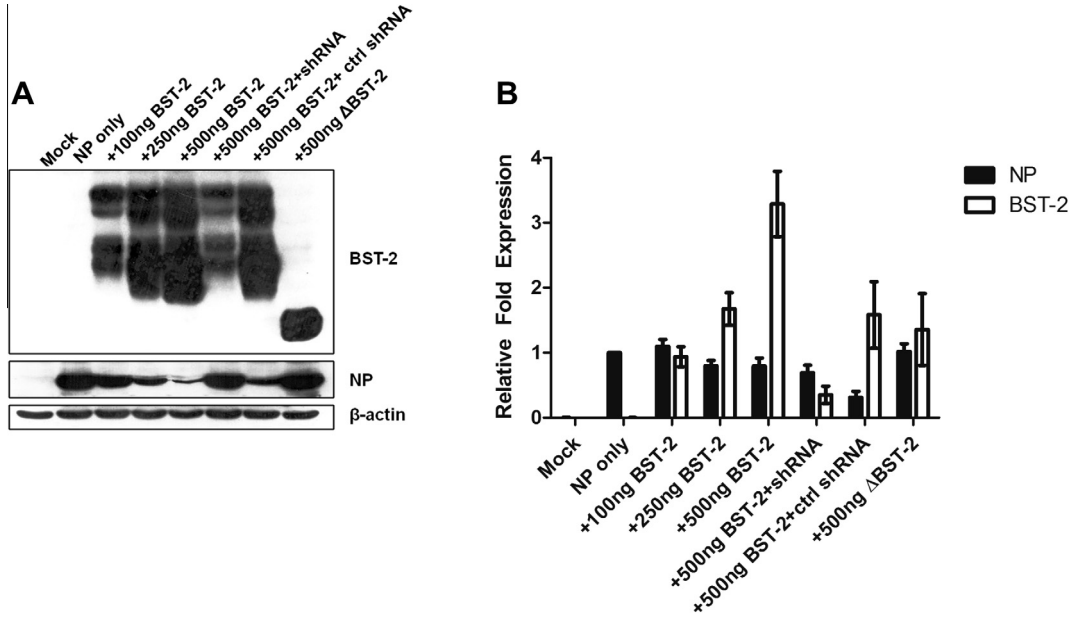

Fig. 2. BST-2 decreases protein levels of transiently expressed NP. HEK293T cells were plated in 6-well plates and transfected with pHW-NP along with the indicated plasmids (500 ng unless otherwise indicated). Cells were harvested after 72 h. (A) Cell lysates were analyzed by SDS–PAGE and Western blotting. (B) RNA extracts were analyzed by quantitative real-time RT-PCR for mRNA levels of NP, BST-2 and fibronectin. NP and BST-2 levels were normalized against fibronectin, and expressed as fold expression relative to the expression level of NP alone. Error bars indicate SEM.

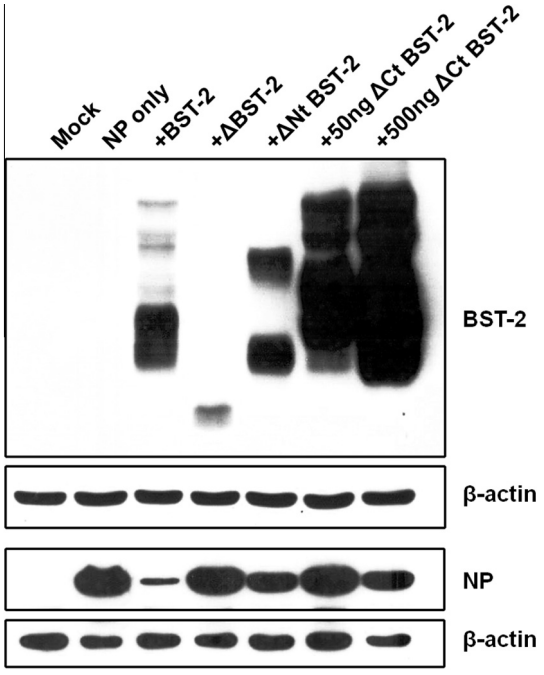

Fig. 3. BST-2 mutants affect NP levels. HEK293T cells were plated in 6-well plates and transfected with pHW-NP and BST-2 wild-type or mutant expression plasmids at 500 ng per plasmid, unless otherwise indicated. After 72 h, transfected cells were harvested and cell lysates were analyzed by SDS–PAGE and Western blotting.

increased levels of NP protein compared to the wild-type BST-2 despite increased levels of ΔCt BST-2 protein.

Finally, we elected to test the specificity of the observed down-regulation of transient protein expression by transfecting pBST-2 along with the luciferase expression vector pEF1α-Luc, and observed a similar negative BST-2-dose-dependent decrease in luciferase activity (Fig. 4). As luciferase expression was being driven by the non-viral human EF1α promoter, this result demonstrated that the impact of BST-2 on transient protein expression was not limited to the NP protein or CMV promoter-driven transcripts alone.

4. Discussion

In this study, we demonstrated the ability of overexpressed BST-2 to mediate down-regulation of transient protein expression. This effect was dose-dependent and specific—the use of anti-BST-2

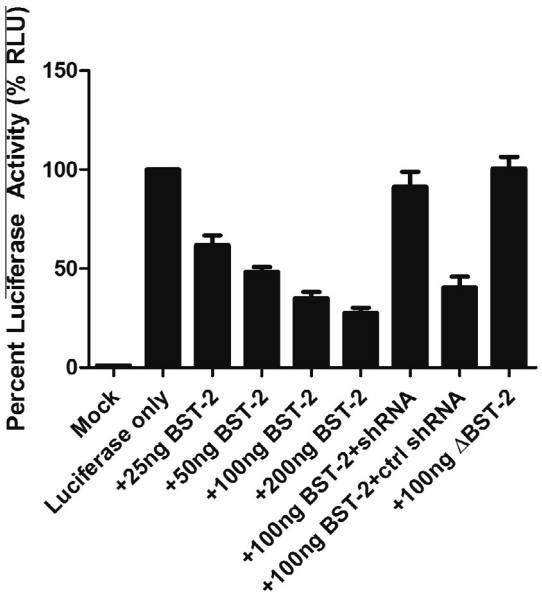

Fig. 4. BST-2 decreases levels of luciferase activity. HEK293T cells were plated in 96-well plates and transfected with pEF1α-Luc along with the indicated expression plasmids. After 48 h, ONE-Glo substrate was added to each well and the resulting luminescence quantified by spectrophotometry. Results are expressed as percent luciferase activity compared to pEF1α-Luc in the absence of BST-2 (Luc only). Error bars indicate SEM. RLU, relative light units.

siRNA or shRNA, and transfection of various BST-2 mutants minimized the impact of BST-2 on NP protein levels. In particular, loss of either the N- or C-terminal domains impaired the down-regulation of transient protein expression, suggesting that this effect may have been mediated by signaling pathways or localization-specific interactions. Furthermore, this effect was not exclusive to a viral promoter or protein; BST-2 also affected activity of luciferase whose expression was driven by the human EF1α promoter.

The ability of BST-2 to affect transient protein expression was a surprising finding. Due to its N-terminal transmembrane domain and a GPI anchor in the C-terminus that enhances localization to membrane lipid rafts [3], BST-2 is membrane-bound and has been found to shuttle between the cell surface membrane and the trans-Golgi network [2,3]. It is therefore unlikely that BST-2 would directly interfere with transcriptional or translational processes.

In recent years, however, several studies have begun to elucidate the signaling capabilities of BST-2, expanding the repertoire of BST-2 as not only an antiviral "tether" but as a viral sensor as well. In particular, BST-2 has been shown to activate the transcription factor NF-κB [22,24,33], which can in turn trigger various antiviral responses by crosstalk with the interferon pathway [35,36]. The N-terminal cytoplasmic tail of BST-2, especially the YxY motif at positions 6 and 8 as well as nearby residues [23,33,34], has been identified as being critical for signaling activity, although mutations in the extracellular and C-terminal domains have also been reported to play a role [23,24]. Crosslinking of BST-2 by virion tethering or antibody binding can trigger activation of the NF-κB pathway as well as overexpression of BST-2 [23,24,34], presumably due to the resultant clustering of BST-2 in the lipid raft microdomains. Alternatively, it has been suggested these lipid raft microdomains themselves may act as signaling platforms, and the role of BST-2 in maintaining and organizing these rafts may therefore be indirectly responsible for signaling activity [20].

Antiviral signaling may activate a number of pathways designed to antagonize viral replication and spread. As our inability to detect a noticeable impact on NP mRNA levels implicates effects at a post-transcriptional level, we surmise that translation dampening mechanisms may be involved. For example, phosphorylation of the translation initiation factor 2 alpha (eIF2α) by the interferon-inducible protein kinase R (PKR) enables inhibition of viral protein synthesis [37–39]. Intriguingly enough, the murine PKR promoter has been characterized as containing NF-κB-like sites [40].

And finally, while the N- and C-termini of BST-2 appear to be important in enabling BST-2-mediated down-regulation of transient protein expression, these two domains are also critical for maintaining the topology of BST-2 generally required for inhibition of viral release. The dual impact of BST-2 on transient protein expression and viral egress during reverse genetics helps to reconcile the differences observed in influenza virus replication levels in the presence of BST-2 during reverse genetics versus infection. The strong impact of BST-2 overexpression on influenza virus yields by reverse genetics points to a possible alternate mechanism by which BST-2 may exert its antiviral effects.

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