Infectious Lassa Virus, but Not Filoviruses, Is Restricted by BST-2/Tetherin[▽]

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Bone marrow stromal antigen 2 (BST-2/tetherin) is a cellular membrane protein that inhibits the release of HIV-1. We show for the first time, using infectious viruses, that BST-2 also inhibits egress of arenaviruses but has no effect on filovirus replication and spread. Specifically, infectious Lassa virus (LASV) release significantly decreased or increased in human cells in which BST-2 was either stably expressed or knocked down, respectively. In contrast, replication and spread of infectious Zaire ebolavirus (ZEBOV) and Lake Victoria marburgvirus (MARV) were not affected by these conditions. Replication of infectious Rift Valley fever virus (RVFV) and cowpox virus (CPXV) was also not affected by BST-2 expression. Elevated cellular levels of human or murine BST-2 inhibited the release of virus-like particles (VLPs) consisting of the matrix proteins of multiple highly virulent NIAID Priority Pathogens, including arenaviruses (LASV and Machupo virus [MACV]), filoviruses (ZEBOV and MARV), and paramyxoviruses (Nipah virus). Although the glycoproteins of filoviruses counteracted the antiviral activity of BST-2 in the context of VLPs, they could not rescue arenaviral (LASV and MACV) VLP release upon BST-2 overexpression. Furthermore, we did not observe colocalization of filoviral glycoproteins with BST-2 during infection with authentic viruses. None of the arenavirus-encoded proteins rescued budding of VLPs in the presence of BST-2. Our results demonstrate that BST-2 might be a broad antiviral factor with the ability to restrict release of a wide variety of human pathogens. However, at least filoviruses, RVFV, and CPXV are immune to its inhibitory effect.

The host innate immune response acts as a first line of defense against viral infections, preventing virus invasion or replication before more specific protection is generated by the adaptive immune system (23). Viral infection or recognition of viral nucleic acids initiates signaling pathways that lead to the synthesis of multiple cytokines, including type I interferons (IFNs), such as IFN- α and IFN- β , which evoke coordinated antiviral responses in the host. Viruses have evolved multiple strategies to counter the IFN system by suppressing IFN production, signaling, or IFN antiviral effector proteins, thereby facilitating infection (23).

Bone marrow stromal antigen 2 (BST-2; also called CD317, HM1.24, or tetherin) is a glycosylphosphatidylinositol-anchored type II transmembrane protein that is upregulated on most cell types upon stimulation with type I IFNs or IFN- γ (8, 24, 33). BST-2 shuttles between the plasma membrane, where it is present predominantly in lipid rafts, and the *trans*-Golgi network, via a clathrin-mediated pathway (33, 55).

Several groups have described the antiviral role of BST-2 as an inhibitor of retrovirus release (28, 49, 63), and demonstrated that human immunodeficiency virus type 1 (HIV-1)

Vpu can antagonize this function. Specifically, cell lines that endogenously expressed BST-2 or were stimulated with IFN- α exhibited markedly decreased budding of retroviral virus-like particles (VLPs) or Vpu-defective HIV-1 compared to BST-2-negative cells. Virions produced in BST-2-expressing cells were retained on the cell surface and subsequently internalized into BST-2-positive endosomal compartments. This restriction could be overcome by HIV-1 Vpu, which downregulates BST-2 from the cell surface via β -TrCP and endolysosomal trafficking (16, 45).

Lake Victoria marburgvirus (MARV), Zaire ebolavirus (ZEBOV), Lassa virus (LASV), Machupo virus (MACV), and Nipah virus (NiV) are highly virulent NIAID Priority Pathogens. Marburgviruses and ebolaviruses, members of the family *Filoviridae*, as well as Lassa and Machupo viruses, members of the family *Arenaviridae*, cause severe hemorrhagic fevers in humans and nonhuman primates with extraordinarily high case-fatality rates (22, 31). NiV, an emerging zoonotic paramyxovirus (15), was implicated as the cause of a rapidly fatal, febrile human encephalitis. Currently, U.S. Food and Drug Administration-approved vaccines or antivirals are not available for preventing or treating the diseases caused by any of these agents (9, 43).

ZEBOV, MARV, LASV, MACV, and NiV are enveloped RNA viruses that egress from their host cells by membrane protrusion (budding). Expression of the matrix proteins of these pathogens—VP40 (ZEBOV and MARV) (6, 25, 60), Z

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(LASV and MACV) (59), or M (NiV) (52, 53)—mimics this process and results in formation of VLPs that are released from the cell surface in a fashion similar to that of the HIV-1 matrix protein Gag (11, 47). These VLPs structurally resemble infectious virions (6, 19, 52) and therefore represent a suitable tool for both molecular-biological studies of viral budding and the development of vaccines (20, 38).

Recent reports have suggested a broad antiviral activity of BST-2. In addition to retroviruses, BST-2 can also inhibit release of VLPs generated by expression of the matrix proteins of ZEBOV, MARV, or LASV (28, 29, 56). Furthermore, the ZEBOV glycoprotein, GP_{1,2}, was found to antagonize the antiviral activity of BST-2 in the context of VLPs, similarly to HIV-1 Vpu (29).

We examined here the hypothesis that BST-2 might be a general inhibitor of enveloped virus release. Only egress of infectious arenaviruses was affected by elevated BST-2 levels. The filovirus-encoded glycoprotein GP_{1,2} could counteract the antiviral activity of BST-2 and rescued replication of infectious filoviruses, whereas none of the arenavirus-encoded proteins antagonized BST-2. We also demonstrate that BST-2 can significantly decrease the release of VLPs of pathogenic viruses from three different families. Specifically, BST-2 overexpression inhibited the release of filoviral (ZEBOV and MARV), arenaviral (LASV and MACV), and paramyxoviral (NiV) VLPs.

MATERIALS AND METHODS

Plasmids, antibodies, and reagents. Genes encoding human or murine FLAG-BST-2, HIV-1 Vpu-V5, ZEBOV (isolate Mayinga) NP-V5, VP35-V5, VP30-V5, V5-VP24, HA-VP40, GP_{1,2}, GP_{1,2}-V5 lacking the mucin-like domain (GP_{1,2}ΔMLD-V5), sGP-V5, ssGP-V5, and Δ-peptide-V5; MARV (isolate Musoke) HA-VP40, MACV (strain Carvalho) Z-HA, NP-V5, and L; LASV (strain Josiah) SSP-V5, Z-HA, and NP-V5; and NiV M-HA were commercially synthesized (Geneart AG or Blue Heron Biotechnology). Plasmid encoding EGFP-VPS4A E228Q was previously described (58). Filoviral HA-VP40, arenaviral Z-HA, and NiV M-HA were cloned into the pCAGGS expression plasmid (54). The remaining genes were cloned into pcDNA3.1/Hygro(+) or pcDNA3.1(+) (Invitrogen). The MACV and LASV GPC expression vectors, as well as MARV GP_{1,2}, were previously described (32, 54). Chloramphenicol acetyltransferase (CAT) antisense was purchased from Invitrogen, and rabbit polyclonal antibody against BST-2 was obtained from Thermo Scientific Open Biosystems.

Cells and transfections. 293T cells (ATCC, CRL-11268) and HeLa cells (ATCC, CCL-2) were maintained in Dulbecco modified Eagle medium and Eagle minimum essential medium, respectively, supplemented with 10% fetal calf serum. 293 cells stably expressing human BST-2, CAT or an empty plasmid (pCDNA5/FRT) were generated by using the Flp-In system according to the manufacturer's instructions (Invitrogen). Stable clones were selected in hygromycin B (100 µg/ml) and analyzed by quantitative reverse transcription-PCR (qRT-PCR) and Western blot assays. 293T and 293/Flp-In cells were transfected by using Lipofectamine 2000 (Invitrogen).

siRNA knockdowns. HeLa cells were transfected in 96-well plates (8,000 cells per well) with 80 nM small interfering RNA (siRNA) targeting human BST-2 (ON-TARGETplus, catalog no. L-011817-00), control siRNA (ON-TARGETplus non-targeting siRNA, catalog no. D-001810-04-05) (Thermo Scientific Dharmacon) or in the absence of siRNA using Lipofectamine 2000 (Invitrogen). Cells were infected with ZEBOV-GFP (61), MARV isolate Ci67, or LASV strain Josiah 24 h later.

VLP release assays. 293T cells in 12-well plates were transfected with 1 µg of plasmid encoding filoviral HA-VP40, arenaviral Z-HA, or NiV M-HA, together with 1 µg of empty vector or vector expressing enhanced green fluorescent protein (EGFP)-VPS4A E228Q or human or murine FLAG-BST-2. Alternatively, 293 cells stably expressing BST-2, CAT, or an empty plasmid were transfected with 2 µg of plasmid encoding filoviral HA-VP40, arenaviral Z-HA, or NiV M-HA. In rescue experiments, 293 cells stably expressing human BST-2 were transfected with plasmids encoding (i) ZEBOV HA-VP40 together with

ZEBOV NP-V5, VP35-V5, VP30-V5, V5-VP24, GP_{1,2}, GP_{1,2}ΔMLD-V5, sGP-V5, ssGP-V5, or Δ-peptide-V5 or HIV-1 Vpu-V5; (ii) MARV HA-VP40 together with MARV GP_{1,2} or HIV-1 Vpu-V5; (iii) MACV Z-HA together with MACV NP-V5, GPC, or L-FLAG or HIV-1 Vpu-V5 or filoviral GP_{1,2}; or (iv) LASV Z-HA together with LASV NP-V5, GPC, or SSP-V5 or HIV-1 Vpu-V5 or filoviral GP_{1,2}. Cells were washed and supplemented with growth medium 2 h posttransfection. Cells and culture supernatants were collected for analysis 48 h later. Culture supernatants from transfected cells were clarified by low-speed centrifugation and passed through a 0.22-µm-pore-size filter (Millipore), and virus particles were pelleted through a 20% sucrose cushion at 22,000 × g at 4°C for 2 h (44, 48, 49, 66). Cells were detached with cell dissociation buffer (Invitrogen), washed with phosphate-buffered saline (PBS), and lysed with radioimmunoprecipitation assay lysis and extraction buffer (Thermo Scientific Pierce) supplemented with Complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 22,000 × g at 4°C for 20 min, and FLAG-, HA-, and V5-tagged proteins were immunoprecipitated with EZview Red Anti-FLAG M2 affinity gel, EZview Red Anti-HA affinity gel, or anti-V5 Agarose affinity gel, respectively (Sigma). Pelleted VLPs and corresponding cell lysate immunoprecipitates were analyzed by SDS-PAGE and Western blotting using Western-Breeze chromogenic kits (Invitrogen) and murine monoclonal anti-FLAG-, anti-HA-, or anti-V5-alkaline phosphatase antibodies (Sigma/Invitrogen). Alternatively, expression of actin, LASV GPC, ZEBOV GP_{1,2}, MARV GP_{1,2}, and VPS4A E228Q was determined using murine monoclonal antibodies against actin (BD Biosciences), LASV GP1 (161-6), ZEBOV GP_{1,2} (6D8), or MARV GP_{1,2} (5D7) or using rabbit polyclonal antibodies against VPS4A (Santa Cruz Biotechnology). Semiquantitative analysis of Western blots was carried out by quantifying band intensities (given in arbitrary units) associated with released VLPs using ImageJ software (W. S. Rasband, ImageJ, U.S. National Institutes of Health [NIH], Bethesda, MD [http://rsb.info.nih.gov/ij/], 1997 to 2009).

Infection assays. siRNA-treated HeLa cells in 96-well plates were infected with LASV Josiah strain (multiplicity of infection [MOI] = 0.1), ZEBOV-GFP (MOI = 10) (61), or MARV isolate Ci67 (MOI = 3). 293 cells stably expressing human BST-2, CAT, or an empty vector in 96-well plates were infected with LASV Josiah strain or ZEBOV isolate Mayinga, at MOIs of 0.5, 1, or 5. The inocula were removed 1 h later, and the cells were washed three times with PBS. Culture supernatants were harvested in TRIzol (Invitrogen) at 48 or 72 h postinfection, and the virion yield was determined by qRT-PCR. Alternatively, cells were fixed in 10% buffered formalin (Val Tech Diagnostics) for 72 h and stained for high-content quantitative image-based analysis with murine monoclonal antibodies against ZEBOV or MARV GP_{1,2} (6D8 or 9G4 antibodies, respectively), followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). Cells were also infected for 1 h with Rift Valley fever virus (RVFV) MP12 vaccine strain or eGFP-expressing cowpox virus (CPXV-GFP) at different MOIs. At 24 or 48 h postinfection, the cells were fixed with formalin for 72 h. RVFV-infected cells were stained with murine monoclonal antibody against the RVFV glycoprotein (4D4) (5, 30), followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). All infected cells were stained with Hoechst 33342 and HCS CellMask Red (Invitrogen).

qRT-PCR. Total RNA from culture supernatants of untreated cells (mock) or cells infected with LASV or ZEBOV was prepared by using a MagMax 96 RNA extraction kit (Ambion). qRT-PCR assays were performed on an ABI Prism 7900HT sequence detection system with the RNA UltraSense one-step kit (Invitrogen) and TaqMan probes (Applied Biosystems) in accordance with the manufacturers' instructions. The final concentrations used in the 20-µl reaction mix contained 5 µl of RNA, 0.4 µM concentrations of each primer, 0.2 µM probe, 4 µl of 5× reaction mix, 0.4 µl of Rox, and 1 µl of enzyme mix. The reaction was run as follows: RT at 50°C for 20 min; initial denaturation at 95°C for 2 min; and then amplification for 40 cycles at 95°C for 15 s and 60°C for 30 s. Serial 10-fold dilutions of the assayed (10² to 10⁷ copies) virus were used as standards. The sequences of the primers and probes used for determination of the virus copy number are listed in Table 1.

To determine the relative expression levels of BST-2 in siRNA-treated HeLa cells, total RNA was isolated 24, 48, and 72 h after transfection by using the RNeasy Plus minikit (Qiagen). Purified RNA was then measured by using the Quant-IT RiboGreen RNA assay kit (Invitrogen). Portions (50 ng) of the RNA were used in qRT-PCR assays using the conditions described above with BST-2 and GAPDH TaqMan probes (Applied Biosystems). Relative expression levels were determined by using the comparative C_T method.

Immunofluorescence and confocal microscopy. For immunofluorescence studies, 293 cells stably expressing human BST-2 were plated on BD BioCoat eight-chamber poly-D-lysine culture slides (Fisher Scientific) and infected with ZEBOV Mayinga isolate or MARV Ci67 isolate at MOIs of 0.5 or 5, respectively. At 24 or 48 h after infection, the cells were fixed in 10% buffered formalin

TABLE 1. Sequences of the primers and probes used for determination of virus copy number in qRT-PCR assays

Primer or probe	Target gene	Sequence
ZEBOV-Forward	<i>GP</i>	TGGGCTGAAAACCTGCTACAATC
ZEBOV-Reverse	<i>GP</i>	CTTTGTGCACATACCGGCAC
ZEBOV-Probe	<i>GP</i>	CTACCAGCAGCGCCAGACGG
LASV-Forward	<i>GPC</i>	GCAGTGCTGAAAGGTCTGTACAA
LASV-Reverse	<i>GPC</i>	AGGAGGAAAGTGACCAAACCAA
LASV-Probe	<i>GPC</i>	TTTGCAACGTGTGGCCT

for 72 h and blocked in 3% bovine serum albumin-PBS for 1 h. BST-2 was stained using rabbit anti-BST-2 antibody (Open Biosystems), followed by Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen). MARV and ZEBOV GP_{1,2} were stained with murine monoclonal antibodies against ZEBOV or MARV GP_{1,2} (6D8 or 9G4 antibodies, respectively), followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). Nuclei were stained with Hoechst 33342. The confocal images were collected on a Leica TCS-SP5 confocal/multiphoton microscope.

High-content quantitative imaging data were acquired and analyzed on an Opera confocal reader (model 3842; quadruple excitation high sensitivity; Perkin-Elmer), at two exposures using a $\times 10$ air objective lens. The first exposure utilized the 488-nm and 640-nm lasers to excite the viral and cell body fluorophores, respectively. The emission light was split by a 580-nm short-pass dichroic mirror and collected on separate cameras through 562/540-nm and 690/670-nm band-pass filters. The second exposure used a 425-nm long-pass mirror to steer the UV (350 nm) to the sample to excite the nuclear stain. The visible emission was directed to the nonconfocal camera by a 475-nm long-pass filter with the emission defined with a 450/450-nm band-pass filter. The optimal z-position for the 488-nm signal was determined by using the delta correction script. Analysis of the images was accomplished within the Opera environment using standard Acapella scripts.

GenBank accession numbers. Sequence data can be found in the GenBank data libraries under accession numbers NM_198095 for murine BST-2, D28137 for human BST-2, P05923 for HIV-1 Vpu, NC_002549 for ZEBOV, DQ217792 for MARV, NC_005079 and AY571904 for MACV, AY628202 and AY628203 for LASV, and NC_002728 for NiV.

RESULTS

LASV, MACV, ZEBOV, MARV, and NiV VLP release is significantly decreased in murine or human BST-2-expressing 293T cells. Expression of the matrix proteins of various enveloped viruses, including filoviruses (ZEBOV and MARV VP40), arenaviruses (LASV and MACV Z), and paramyxoviruses (NiV M), results in the formation of VLPs that are released from the cells in a similar fashion to HIV-1 Gag (6, 25, 52, 53, 59, 60). To examine the ability of BST-2 to inhibit budding of such VLPs, 293T cells, in which expression levels of endogenous BST-2 are minimal (49), were transiently transfected with HA-tagged arenaviral Z, filoviral VP40, or NiV M, together with FLAG-tagged human or murine BST-2. Transfection of an empty expression vector was used as a negative control, and transfection of a vector expressing an enzymatically inactive VPS4A mutant (E228Q) (21) was used as positive control since this cellular AAA-type ATPase has previously been demonstrated to be involved in budding of different enveloped viruses (21, 34, 37, 58, 62). Release of VLPs and expression levels of the different matrix proteins in cell lysates were evaluated by Western blot analysis. As shown in Fig. 1A, expression of either murine or human BST-2 significantly decreased release of ZEBOV, MARV, LASV, MACV, and NiV VLPs. This inhibitory effect was not due to major differences in expression levels of the different matrix proteins (Fig. 1B).

Furthermore, as expected, VPS4A E228Q decreased budding of the different VLPs to various extents with the exception of NiV, as previously reported (53). Figure 1B shows the expression levels of human and murine BST-2, as well as VPS4A E228Q and actin (loading control). Of note, BST-2 appears as multiple bands on the gel with estimated sizes of 21 to 35 kDa, presumably because of heterogeneous glycosylation (24).

To further study the effect of BST-2 on viral release, we established 293 cell lines stably expressing human BST-2, CAT or an empty plasmid. Again, stable expression of human BST-2 inhibited the budding of ZEBOV, MARV, LASV, MACV, and NiV VLPs, whereas expression of CAT had no effect on VLP release (Fig. 2A). Similar expression levels of the different matrix proteins were observed in the cell lysates and could not account for the observed differences in VLP release (Fig. 2B). These data demonstrate the broad antiviral effects of both human and murine BST-2, which can inhibit release of VLPs of various human pathogens belonging to three viral families.

Filoviral GP_{1,2} but not arenaviral GPC counteracts BST-2 antiviral activity. Filoviruses, which are negative-strand RNA viruses, encode six genes in addition to VP40 (NP, VP35, GP, VP30, VP24, and L). Cotranscriptional RNA editing of the ebolavirus, but not MARV, GP gene produces four proteins: GP_{1,2}, sGP, ssGP, and Δ -peptide (31, 57, 64, 65). We sought to examine whether one of the ebolavirus-encoded proteins has a Vpu-like function and could rescue VLP release from cells that overexpress BST-2. To this end, we utilized a 293 cell line stably expressing human BST-2. Cells were transfected with plasmid encoding the matrix protein of ZEBOV (VP40), together with plasmids encoding other ebolavirus proteins (NP, VP35, VP30, VP24, GP_{1,2}, sGP, ssGP, or Δ -peptide). Transfection of a plasmid expressing HIV-1 Vpu or an empty plasmid served as controls. As expected, release of ZEBOV VLPs was promoted by HIV-1 Vpu (Fig. 3A) (29). Of the eight Ebolavirus proteins, only GP_{1,2} rescued ZEBOV VLP release to levels similar to those observed in Vpu-expressing cells. However, as GP_{1,2} expression was previously shown to enhance the efficiency of VP40-driven VLP release in 293T cells (36, 50), we wanted to examine whether ZEBOV GP_{1,2} is a bona fide BST-2 antagonist or whether the observed increase in VLP production is due to coexpression of VP40 and GP_{1,2}. To this end, we compared the increase in VLP release between 293 cells that express minimal levels of BST-2 (FLP) and 293 cells stably expressing BST-2 (FLP-BST-2). The increase in ZEBOV VLP production upon expression of GP_{1,2} in FLP-BST-2 was significantly higher than that observed in FLP cells, indicating that ZEBOV GP_{1,2} is indeed a BST-2 antagonist (Fig. 4).

We also tested a GP_{1,2} deletion variant lacking the mucin-like domain (GP_{1,2} Δ MLD) for its ability to function as a BST-2 antagonist. This variant was almost as potent as wild-type GP_{1,2} in promoting ZEBOV VLP release, suggesting that the mucin-like domain is most likely dispensable for this function (Fig. 3A). NP, VP35, and VP24 were previously reported to influence viral release (27, 36). In this assay, however, their effect was less pronounced compared to GP_{1,2} (Fig. 3A). Expression of ZEBOV VP40, as well as the different ebolavirus proteins and HIV-1 Vpu in the cell lysates, are shown in Fig. 3B (upper and bottom panels, respectively).

We next examined whether MARV GP_{1,2} was also capable

FIG. 1. BST-2 inhibits budding of arenaviral, filoviral, and paramyxoviral VLPs. (A) Human 293T cells were cotransfected with plasmids encoding hemagglutinin (HA)-tagged matrix proteins of LASV, MACV, ZEBOV, MARV, or NiV, together with an empty plasmid, or plasmid encoding VPS4A-E228Q, human BST-2 (hBST-2), or murine BST-2 (mBST-2). Cell lysates and supernatants were harvested 48 h after transfection. VLPs in clarified medium were pelleted through a sucrose cushion, and HA-tagged matrix proteins in VLPs were analyzed by Western blotting. Numbers below each lane indicate values obtained with densitometric scanning using the ImageJ program (NIH). (B) Expression of the HA-tagged matrix proteins, actin, VPS4A-E228Q, hBST-2, or mBST-2 in cell lysates was determined by Western blotting. Shown is a representative Western blot from three independent experiments.

of rescuing VP40-mediated VLP release in this system. 293 cells stably expressing human BST-2 were transfected with a plasmid encoding MARV VP40, together with plasmids encoding MARV GP_{1,2} or HIV-1 Vpu, or an empty plasmid. As shown in Fig. 3A, MARV GP_{1,2}, akin to ZEBOV GP_{1,2}, could promote MARV VLP release to levels similar to those ob-

served in Vpu-expressing cells. Overall, these results confirm that filoviral GP_{1,2} acts as a BST-2 antagonist (29). We also tested arenavirus-encoded proteins for their ability to counteract BST-2. In addition to Z, arenaviruses encode three other genes: NP, GPC, and L (10, 13, 26). The arenaviral envelope glycoprotein (GPC) is the filoviral GP_{1,2} counterpart.

FIG. 2. Release of arenaviral, filoviral, and paramyxoviral VLPs is reduced in cells stably expressing human BST-2. (A) Human 293 cells stably expressing human BST-2 (FLP-BST-2), CAT (FLP-CAT), or an empty plasmid (pCDNA5/FRT, FLP) were transfected with plasmids encoding HA-tagged matrix proteins of LASV, MACV, ZEBOV, MARV, or NiV. Cell lysates and supernatants were treated as in Fig. 1, and HA-tagged matrix proteins in VLPs were analyzed by Western blotting. Numbers below each lane indicate values obtained with densitometric scanning using the ImageJ program (NIH). (B) Expression of the HA-tagged matrix proteins and actin in cell lysates was determined by Western blot analysis. Shown is a representative Western blot from three independent experiments.

The stable signal peptide (SSP) of GPC plays unique roles in the proteolytic maturation of GPC, intracellular trafficking of GPC from the ER to the cell surface, and arenaviral pH-dependent membrane fusion during entry (1, 17, 18). In contrast to filoviral GP_{1,2}, arenaviral GPC did not promote arenaviral VLP release (Fig. 3C). Similar results were obtained with the other arenavirus-encoded proteins (LASV NP and SSP, and MACV NP and L), which, compared to HIV-1 Vpu, had less or no effect on VLP release. The expression of arenaviral Z in cell lysates is shown in Fig. 3D (upper panel), as well as the expression of other arenavirus-encoded proteins (lower panel). These results demonstrate that, unlike filoviruses and retroviruses, arenaviruses do not encode a BST-2 antagonist.

Finally, we wanted to examine whether filoviral GP_{1,2}, similarly to HIV-1 Vpu, is a general BST-2 antagonist that can overcome the BST-2 antiviral effect not only in the context of filovirus release but also in the context of release of other BST-2-sensitive viruses. To this end, we tested the ability of filoviral GP_{1,2} to rescue arenavirus VLP production when BST-2 is expressed and compared it to HIV-1 Vpu. 293 cells stably expressing human BST-2 were transfected with plasmids encoding the matrix protein of arenaviruses (LASV or MACV Z), together with an empty plasmid or plasmid encoding HIV-1 Vpu, ZEBOV GP_{1,2}, or MARV GP_{1,2}. HIV-1 Vpu promoted arenavirus VLP release, as expected, whereas the filoviral glycoprotein had less or no effect on MACV or LASV VLP release, respectively (Fig. 5). These results suggest that, in

the context of arenavirus VLP release, the filoviral glycoprotein cannot counteract the antiviral activity of BST-2. Of note, expression of all matrix proteins in cell lysates was reduced whenever HIV-1 Vpu was expressed. We believe that this is because Vpu enhances matrix protein release as VLPs.

Effect of human BST-2 overexpression on the release and spread of infectious LASV, ZEBOV, RVFV, and CPXV. VLPs are a valuable system for initial studies of viral budding. However, the system is simplistic and uses only one or few viral proteins in the absence of other virus-encoded proteins or genomic elements. Therefore, any results obtained with this system should not be extrapolated until verified with infectious virus. To this end, 293 cell lines stably expressing human BST-2, CAT, or an empty plasmid were infected with one representative virus of the family *Arenaviridae* (LASV) or one representative virus of the family *Filoviridae* (ZEBOV) at different MOIs. Viral RNA was extracted from the media, and the viral copy number was determined by qRT-PCR. Alternatively, the cells were fixed in formalin and stained for high-content quantitative image-based analysis with virus-specific antibodies. Stable BST-2 expression reduced LASV virion yield by >10-fold, whereas CAT expression had no effect on LASV copy number (Fig. 6A). Conversely, BST-2 expression had no effect on replication and the spread of infectious ZEBOV (Fig. 6B) or on virus release into the supernatant, as determined by qRT-PCR (data not shown).

We then evaluated the antiviral activity of human BST-2

FIG. 3. Filoviral GP_{1,2}, but none of the arenavirus-encoded proteins, counteract human BST-2. (A) Human 293 cells stably expressing human BST-2 were cotransfected with ZEBOV HA-VP40 DNA and plasmids encoding ZEBOV VP30-V5, VP35-V5, V5-VP24, GP_{1,2}, GP_{1,2}ΔMLD-V5, sGP-V5, ssGP-V5, Δ-peptide-V5, or NP-V5 or HIV-1 Vpu-V5. Alternatively, cells were transfected with MARV HA-VP40 DNA, together with plasmids encoding MARV GP_{1,2} or HIV-1 Vpu-V5. Cell lysates and supernatants were treated as in Fig. 1, and HA-tagged VP40 in VLPs were analyzed by Western blotting. Numbers below each lane indicate values obtained with densitometric scanning using the ImageJ program (NIH). (B) Expression of HA-tagged VP40, actin, and other filovirus-encoded proteins in cell lysates was determined by Western blotting. Expression of HIV-1 Vpu (~16 kDa), ZEBOV VP30 (~30 kDa), VP35 (~35 kDa), VP24 (~24 kDa), GP_{1,2}ΔMLD (~75 kDa), sGP (~50 kDa), ssGP (~47 kDa), Δ-peptide (~10 kDa), and NP (~104 kDa) was detected using anti-V5 antibody. Expression of ZEBOV (~140 kDa) and MARV (~170 kDa) GP_{1,2} was detected using anti-GP antibodies (6D8 and 5D7, respectively). (C) Human 293 cells stably expressing human BST-2 were cotransfected with LASV Z-HA DNA and plasmids encoding LASV NP-V5, SSP-V5, or GPC or HIV-1 Vpu-V5. Alternatively, cells were transfected with MACV Z-HA and MACV NP-V5, GPC, L-FLAG, or HIV-1 Vpu-V5. Cell lysates and supernatants were treated as in Fig. 1, and HA-tagged Z proteins in VLPs were analyzed by Western blotting. Numbers below each lane indicate values obtained with densitometric scanning using the ImageJ program (NIH). (D) Expression of HA-tagged Z, actin, and other arenavirus-encoded proteins in cell lysates was determined by Western blotting. Expression of HIV-1 Vpu (~16 kDa), arenaviral NP (~63 kDa), and LASV SSP (~10 kDa) was detected by using anti-V5 antibody. Expression of MACV L-FLAG (~200 kDa) was detected using anti-FLAG antibody. Expression of LASV GPC (~75 kDa) and GP1 (~42 kDa) was detected with an antibody against GP1 (161-6). Shown is a representative Western blot from three independent experiments.

against RNA viruses that lack a structural matrix protein or DNA viruses. The effect of BST-2 overexpression on replication of RVFV, a representative RNA virus of the family *Bunyaviridae*, and on cowpox virus (CPXV), a representative DNA virus of the family *Poxviridae*, was examined. As shown in Fig. 6C and D, neither RVFV nor CPXV replication and spread was significantly affected by BST-2 overexpression.

To summarize, in the context of the viruses tested here, only infectious arenaviruses (LASV) are restricted by human BST-2. These results are not surprising since filoviral GP_{1,2} can counteract BST-2 antiviral activity, whereas no arenavirus-encoded protein has such a function (Fig. 3).

Effect of human BST2 knockdown on the release and spread of infectious LASV, ZEBOV, and MARV. To further verify our

overexpression studies, we chose an alternative and complementary siRNA knockdown approach to test BST-2's antiviral activity. To this end, HeLa cells, which endogenously express high levels of BST-2 (49), were transfected with siRNA targeting BST-2 or a control siRNA. As shown in Fig. 7A, the expression of human BST-2 was effectively knocked down 24, 48, or 72 h posttransfection. siRNA targeting BST-2, but not control siRNAs, enhanced the yield of LASV from HeLa cells by almost 2-fold (Fig. 7B). In contrast, only negligible effects on filovirus replication and spread were observed under these conditions (Fig. 7C). These results further demonstrate that in the context of infectious viruses, BST-2 restricts only arenaviruses (LASV) and not filoviruses.

Localization of human BST2 and filoviral GP_{1,2} during filovirus infection. To begin to delineate the mechanism by which

FIG. 4. The increase in ZEBOV VLP release when GP_{1,2} is expressed in the presence of BST-2 is significantly higher than that observed in the absence of BST-2. (A) Human 293 cells stably expressing human BST-2 (FLP-BST-2) or an empty plasmid (FLP) were transfected with ZEBOV VP40, together with an empty plasmid or a plasmid encoding GP_{1,2}, or HIV-1 Vpu. Cell lysates and supernatants were treated as in Fig. 1, and HA-tagged VP40 in VLPs was analyzed by Western blotting. Numbers below each lane indicate values obtained with densitometric scanning using the ImageJ program (NIH). (B) Expression of the HA-tagged VP40, actin, GP_{1,2}, and HIV-1 Vpu-V5 in cell lysates was determined by Western blot analysis. Shown is a representative Western blot from three independent experiments.

filoviral GP_{1,2} antagonizes BST-2, 293 cells stably expressing human BST-2 were infected with MARV or ZEBOV for 24 or 48 h and stained for immunofluorescence analysis with antibodies against BST-2 and antibodies against ZEBOV or MARV GP_{1,2}. Colocalization of BST-2 with ZEBOV or MARV GP_{1,2} was not observed under any of these conditions (Fig. 8). Instead, BST-2 and the filoviral glycoprotein exhibited complementary distribution at the plasma membrane. Areas with the highest BST-2 staining showed the lowest filoviral GP_{1,2} staining and vice versa.

DISCUSSION

Previous studies demonstrated restriction of ZEBOV, MARV, and LASV VLP release by BST-2 using minimal viral components (28, 29, 56). However, none has examined the effect of BST-2 expression levels on the release and spread of infectious viruses. We found that knockdown or overexpression of BST-2 increased and decreased infectious LASV release, respectively (Fig. 6A and Fig. 7B). These results are in accordance with our data showing that arenaviruses do not encode a BST-2 antagonist (Fig. 3). It is interesting to speculate how arenaviruses overcome BST-2 restriction in the absence of a direct antagonism of its function. Since BST-2 is an

FIG. 5. Expression of filoviral GP_{1,2} has little or no effect on release of arenaviral VLPs in BST-2-expressing cells. (A) Human 293 cells stably expressing human BST-2 were cotransfected with arenaviral Z-HA DNA and an empty plasmid or plasmids encoding filoviral GP_{1,2} or HIV-1 Vpu-V5. Cell lysates and supernatants were treated as in Fig. 1, and HA-tagged Z proteins in VLPs were analyzed by Western blotting. (B) Expression of HA-tagged Z, actin, HIV Vpu-V5, and filoviral GP_{1,2} in cell lysates was determined by Western blotting. Shown is a representative Western blot from three independent experiments.

effector protein of the IFN-induced antiviral response, one possibility is that arenaviruses target BST-2 indirectly by attenuating type I IFN production. Indeed, Martínez-Sobrido et al. showed that arenaviral nucleoprotein (NP) could inhibit the induction of type I IFN (41, 42), which could in turn prevent stimulation of BST-2 expression. Furthermore, it is plausible that arenaviruses target cells that express low levels of BST-2 in the absence of IFN induction. Human dendritic cells, macro-

FIG. 6. Human BST-2 overexpression inhibits infectious LASV, but not ZEBOV, release and spread. (A) Human 293 cells stably expressing human BST-2 (FLP-BST-2), CAT (FLP-CAT), or an empty plasmid (FLP) were infected with LASV at the indicated MOIs. After 72 h, viral RNA was extracted from the media, and the viral copy number was determined by qRT-PCR. (B to D) Alternatively, cells were infected with ZEBOV (B), CPXV-GFP (C), or RVFV (D), fixed in formalin and stained for high-content quantitative image-based analysis with virus-specific antibodies 24 or 48 h (C and D) or 72 h (B) after infection. Error bars indicate standard deviations.

phages, and endothelial cells are known to be such arenaviral targets (2, 39). BST-2 expression is low to undetectable in these cells but can be induced by IFN- α (12, 46). Therefore, we hypothesize that arenaviruses target cells that express minimal levels of BST-2 and prevent its further induction by NP-mediated inhibition of type I IFN production. Thus, even in the absence of a direct BST-2 antagonist, arenaviruses could still multiply in their host(s) with minimal deleterious effects of this antiviral protein. Several observations support this hypothesis and show that type I IFNs are crucial in the control of arenaviruses. First, whereas especially high arenavirus titers could be obtained from infected dendritic cells, lower titers were obtained from infected macrophages. In accordance with this, no type I IFN production was observed in dendritic cells, whereas

a moderate response was observed in macrophages. In addition, exogenous activation of arenavirus-infected macrophages or dendritic cells, as well as recombinant type I IFN, strongly inhibited viral replication (3).

We also show that BST-2 inhibits the release of a variety of VLPs, generated by the expression of arenaviral (LASV and MACV), filoviral (ZEBOV and MARV), or paramyxoviral (NiV) matrix proteins (Fig. 1 and 2). BST-2's antiviral activity against retroviral VLPs and Vpu-deficient HIV-1 has been demonstrated previously (28, 49, 63). The matrix proteins of these divergent viruses show no sequence homology and are targeted to the cell membrane by different mechanisms. The only commonality shown for filoviruses, NiV, and retroviruses is that all assemble and bud from plasma membrane microdo-

FIG. 7. Knockdown of human BST-2 expression enhances infectious LASV release, but not ZEBOV or MARV spread. (A) HeLa cells were transfected with siRNA targeting BST-2 or with a control siRNA. After 24, 48, or 72 h, cellular RNA was extracted, and the relative BST-2 expression levels were determined by qRT-PCR. BST-2 protein expression levels in cell lysates were also determined by Western blotting. (B) Cells were infected with LASV (MOI = 0.1) 24 h after transfection. Viral RNA was extracted from the medium 72 h later, and the viral copy number was determined by qRT-PCR. (C) Alternatively, cells were infected with ZEBOV-GFP (MOI = 10) or MARV (MOI = 3). Cells were fixed in formalin 72 h later and stained for high-content quantitative image-based analysis with virus-specific antibodies. Error bars indicate standard deviations.

mains termed lipid rafts or detergent-resistant membranes (6, 7, 14, 35, 51). BST-2 also localizes to lipid raft microdomains (33). Overall, these data suggest that BST-2 is a broad non-specific inhibitor of VLP release, whose effect might depend on colocalization with nascent virions in plasma membrane lipid rafts.

We found that filoviral GP_{1,2}, similar to HIV-1 Vpu, rescued VP40-mediated VLP release from cells overexpressing human BST-2 (Fig. 3A). These results are in agreement with our studies with infectious filoviruses, showing that various expression levels of BST-2 had no or minimal effect on filoviral spread (Fig. 6B and Fig. 7C) and release (data not shown). Note that none of our assays demonstrated that filoviral GP_{1,2} counteracted BST-2 directly. Although our confocal microscopy studies show complementary staining of BST-2 and filoviral GP_{1,2} rather than colocalization, they do not necessarily rule out the possibility of such interaction (Fig. 8). A recent study by Kaletsky et al. (29) demonstrated that, reminiscent of HIV-1 Vpu (16, 49), ebolaviral GP_{1,2} indeed specifically interacts with BST-2. Nevertheless, the mechanism by which this filoviral protein antagonizes BST-2 remains under investigation. In the case of HIV-1 Vpu, several studies demonstrated that Vpu directs the degradation of human BST-2 via a β -TrCP-dependent mechanism. Vpu acts as an adapter mole-

cule linking BST-2 to the β -TrCP/SCF E3 ubiquitin ligase complex to induce endolysosomal trafficking and subsequent lysosomal BST-2 degradation. Through this action, HIV-1 Vpu removes BST-2 from its site of action as a virion-tethering factor (16, 45). Studies are under way to determine whether filoviral GP_{1,2} also functions via a β -TrCP-dependent mechanism. If filoviral GP_{1,2}, akin to HIV-1 Vpu, overcomes BST-2 restriction by a mechanism involving BST-2 degradation, a rather transient interaction between these two proteins could be expected that would be rather hard to detect. Such a mechanism would explain why we did not observe BST-2 where filoviral GP_{1,2} is localized: BST-2 in these areas might have been degraded, whereas in membrane domains where filoviral GP_{1,2} is absent, BST-2 could still be present.

Replication and spread of RVFV, a representative bunyavirus that lacks a matrix protein, and CPXV, a representative DNA virus, were not affected by human BST-2 overexpression (Fig. 6C and Fig. 6D). It is possible that, like HIV-1, SIV, and filoviruses (29, 49, 63, 66), these viruses encode BST-2 antagonists that rescue viral release from BST-2-mediated restriction. Recently, a DNA virus, human herpesvirus 8 (HHV-8; also known as Kaposi's sarcoma-associated virus), has been shown to encode such a BST-2 antagonist. The HHV-8-encoded K5 protein is a RING-CH

FIG. 8. BST-2 localization in relation to filoviral GP_{1,2}. (A) Human 293 cells stably expressing human BST-2 were infected with ZEBOV. Cells were fixed in formalin 24 h later and stained with antibodies against BST-2 and ZEBOV GP_{1,2}. (B and C) Alternatively, cells were infected with MARV for 24 h (B) or 48 h (C) and stained with antibodies against BST-2 and MARV GP_{1,2}. Immunofluorescence of anti-filoviral GP_{1,2} (green) and anti-BST-2 (red) in a single confocal plain is shown as indicated. Right columns show the merged images for triple staining of BST-2 (red), filoviral GP_{1,2} (green), and the nucleus (blue), as well as bright-field images.

ubiquitin ligase that ubiquitinylates BST-2, leading to its rapid degradation (4, 40). Furthermore, HHV-8 release is decreased in the absence of K5 in a BST-2-dependent manner, suggesting that BST-2's antiviral activity is not limited to RNA viruses. It would be of interest to examine whether other DNA viruses, such as poxviruses, encode for similar BST-2 antagonists. Alternatively, it is plausible that RVFV and CPXV bud from membranes or membrane domains that lack BST-2. Such a scenario might indicate that the intrinsic properties of viral structural proteins, in particular the specific membrane microdomains to which they are targeted

and where assembly and budding takes place, might influence BST-2 sensitivity.

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