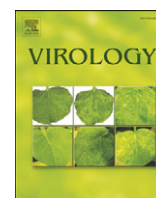Contents lists available at ScienceDirect

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BST-2 mediated restriction of simian–human immunodeficiency virus

Autumn Ruiz [a], David Lau [c], Richard S. Mitchell [c], M. Sarah Hill [b], Kimberly Schmitt [a], John C. Guatelli [c,d], Edward B. Stephens [a,b,*]

[a] Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas 66160, USA
[b] Department of Microbiology, Molecular Genetics, and Immunology, University of Kansas Medical Center, Kansas City, Kansas 66160, USA
[c] Department of Medicine, University of California San Diego, California 92093, USA
[d] The Veterans Affairs San Diego Healthcare System, San Diego, CA 92161, USA

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ABSTRACT

Pathogenic simian–human immunodeficiency viruses (SHIV) contain HIV-1 Vpu and SIV Nef, both shown to counteract BST-2 (HM1.24; CD317; tetherin) inhibition of virus release in a species-specific manner. We show that human and pig-tailed BST-2 (ptBST-2) restrict SHIV. We found that sequential "humanization" of the transmembrane domain (TMD) of the pig-tailed BST-2 (ptBST-2) protein resulted in a fluctuation in sensitivity to HIV-1 Vpu. Our results also show that the length of the TMD in human and ptBST-2 proteins is important for BST-2 restriction and susceptibility to Vpu. Taken together, our results emphasize the importance of tertiary structure in BST-2 antagonism and suggests that the HIV-1 Vpu transmembrane domain may have additional functions in vivo unrelated to BST-2 antagonism.

Introduction

Human bone marrow stromal cell antigen 2 (BST-2; HM1.24; CD317 or tetherin) was recently identified as a potent inhibitor of the release of multiple enveloped viruses including human immunodeficiency virus type I (HIV-1) and simian immunodeficiency virus (SIV) (Jouvenet et al., 2009; Kaletsky et al., 2009; Neil et al., 2008; Van Damme et al., 2008). While the exact cellular function of human BST-2 (hBST-2) in the human host is unclear, it has been shown to play a role in regulating the growth and development of B cells, to be involved in the organization of the subapical actin cytoskeleton in polarized epithelial cells (Rollason et al., 2009), and most recently to function as a ligand for ILT7, a receptor that inhibits IFN production from plasmacytoid dendritic cells (Cao et al., 2009). BST-2 is an interferon-induced, lipid raft-associated, type II integral membrane protein with an unusual topology that is similar in membrane orientation to a neuro-pathogenic form of the prion protein (PrP) (Kupzig et al., 2003). It contains a short cytoplasmic N-terminus followed by a transmembrane domain (TMD), a central extracellular domain

predicted to form a coiled-coil structure, and a C-terminal cleavage site predicted to form a glycosyl-phosphatidylinositol (GPI) anchor (Ishikawa et al., 1995; Kupzig et al., 2003). This topology supports membrane-spanning models of restriction of virion release, in which the protein would form dimers providing a physical, protease-sensitive link between the cellular and viral membranes (Perez-Caballero et al., 2009). This model is supported by evidence that hBST-2 is incorporated into nascent virions and that the formation of cysteine-linked dimers is required for hBST-2 restriction of HIV-1 virion release (Ali et al., 2010; Andrew et al., 2009; Fitzpatrick et al., 2010; Habermann et al., 2010; Perez-Caballero et al., 2009).

Several studies have provided evidence that viruses including HIV-1 and SIV have evolved to acquire activity against the anti-viral properties of BST-2. The HIV-1 group M Vpu proteins counteract specifically the restriction of human, chimpanzee and gorilla BST-2 proteins, with the exception of Vpu from strains JR-CSF and YU-2, which also antagonized BST-2 proteins derived from Greater-spot nosed and African green monkeys (Jouvenet et al., 2009; Neil et al., 2008; Sauter et al., 2009; Van Damme et al., 2008). The Nef proteins isolated from SIV_{mac}, SIV_{cpz}, SIV_{gor}, and SIV_{agm} also antagonize the effects of certain non-human primate BST-2 proteins, including those isolated from rhesus (rhBST-2) and pig-tailed (ptBST-2) macaques (Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009). Similar to other known intrinsic viral restriction factors such as the APOBEC3 and TRIM families of proteins, the ability of specific viral

* Corresponding author. Department of Microbiology, Molecular Genetics, and Immunology University of Kansas Medical Center, Kansas City, Kansas 66160, USA.
E-mail address: estephen@kumc.edu (E.B. Stephens).

proteins to counteract BST-2 restriction is species-specific (Gupta et al., 2009; Jia et al., 2009; McNatt et al., 2009; Zhang et al., 2009). Specific domains and amino acids within these proteins mediate this specificity, allowing small evolutionary changes to confer susceptibility to cross-species infection (Gupta et al., 2009; Jia et al., 2009; McNatt et al., 2009; Yap et al., 2005; Zhang et al., 2009). The interaction of Vpu with hBST-2 involves the TMDs of both proteins (Douglas et al., 2009; McNatt et al., 2009; Rong et al., 2009). Several studies have identified amino acids within the human, rhesus and tantalus monkey BST-2 proteins required for Vpu sensitivity/resistance. These studies found several combinations of substitutions within the transmembrane domain of hBST-2 that reduced susceptibility to Vpu, including delGI,T45I (where glycine and isoleucine residues at positions 25 and 26 were deleted in combination with the substitution of a threonine at position 45 with an isoleucine) and delGI, I33V, I36L (where the glycine and isoleucine residues at positions 25 and 26 were deleted in combination with the substitution of the isoleucine at position 33 with a valine and mutation of the isoleucine at position 36 to a leucine). These studies also noted the importance of the proline residue at position 40 in sensitivity to Vpu (McNatt et al., 2009). More recent studies focused on the specific role of amino acids within the BST-2 proteins from non-human primates in the susceptibility and resistance to SIV$_{mac}$ Nef proteins. Several groups obtained similar results that identified an amino acid motif that is present within the cytoplasmic domain (D/GIWK$_{14–17}$) of pig-tailed and rhBST-2 as important for SIV Nef susceptibility. These investigators showed that this motif could be inserted into the hBST-2 protein and impart susceptibility to the SIV Nef protein (Jia et al., 2009; Zhang et al., 2009).

Simian–human immunodeficiency viruses (SHIVs) have been used extensively for *in vivo* studies to understand the role of HIV-1 Vpu and Env proteins as well as other SIV proteins in viral pathogenesis. These chimeric viruses express both the HIV-1 Vpu and SIV$_{mac}$ Nef proteins shown to counteract BST-2 orthologues from human and non-human primates and possibly represent a useful virus to study these interactions. In this study, we examined the characteristics and anti-viral activities of BST-2 proteins from humans and pig-tailed macaques with specific amino acid exchanges in the context of SHIVs lacking the HIV-1 *vpu* gene, the SIV *nef* gene, or both. Our results indicate that the transmembrane domain is a determinant of HIV-1 Vpu susceptibility, however, the length of the domain may play more of a role in this rather than specific amino acids and or combinations of residues.

Results

Comparison of the sequence of pig-tailed and rhesus bst-2 genes

We amplified and sequenced the *bst-2* genes from five rhesus macaques (CX54, AH64, AS05, AS34 and AS89) and seven pig-tailed macaques (CC8X, W004, W005, W006, W007, W013, and W018). The genes isolated from the rhesus macaques exhibited considerable variability compared to genes isolated from pig-tailed macaques, most notably in the N-terminal regions (Fig. 1). We observed a 50:50 distribution of arginine and cysteine at position 9, a 60:40 ratio of aspartic acid to glycine at position 14, a 70:30 distribution of valine to isoleucine at position 29 and finally an 80:20 ratio of leucine to proline at position 43. In contrast the pig-tailed sequences did not show significant variability with the exception of one sequence that had a leucine instead of a methionine at position 13. Based on the sequence analysis, we cloned the *ptbst-2* gene for expression studies.

Analysis of human and ptBST-2 mutants

We constructed a series of mutations in the human (hBST-2) and pig-tailed macaque (ptBST-2) *bst-2* genes. These are presented in Fig. 2. We first examined the expression of each mutant BST-2 protein by transfection of 293 cells with vectors expressing each mutant

protein and subsequent Western blot analysis of the cell lysates (Fig. 3). Substitution of the leucine and glycine residues at positions 24 and 25 in hBST-2 with alanines or isoleucines (hBST-2LG/AA or hBST-2LG/II) consistently resulted in higher levels of expression, suggesting that these mutations may increase the half-life of these proteins. The only other protein with altered expression was the ptBST-2(ΔDDWIK/+LG). This amino acid substitution resulted in decreased protein expression, which could be due to altered stability of the protein and was not further studied.

SHIV can serve as models for studying BST-2 mediated restriction

In order to examine the ability of the wild-type hBST-2 and ptBST-2 proteins to restrict virus release, we used parental SHIV$_{KU-2MC4}$ to construct three SHIVs that did not express either HIV-1 Vpu (SHIV$_{\Delta Vpu}$), SIV Nef (SHIV$_{\Delta Nef}$) or both proteins (SHIV$_{\Delta Vpu/\Delta Nef}$). HEK 293 cells were co-transfected with plasmids expressing one of the four SHIVs and each of the BST-2 constructs. At 48 h post-transfection, the supernatants were collected and cleared by centrifugation, the cells were lysed and the nuclei and cellular debris cleared by centrifugation. The supernatants and cell lysates were used to quantify the percent p27 antigen release from cells and the supernatants were also evaluated for the number of infectious doses (TCID$_{50}$) (Fig. 4). The percent release of p27 in the context of each SHIV in the presence and absence of each BST-2 protein is shown in Fig. 4A. All samples were normalized to their respective SHIV empty vector controls. Significance in the restriction of p27 release for the SHIV$_{KU-2MC4}$ samples was determined with respect to the parental SHIV$_{KU-2MC4}$ empty vector control using a Student's t-test (▲). Significance in the restriction of p27 release for the SHIV$_{\Delta Vpu}$, SHIV$_{\Delta Nef}$, and SHIV$_{\Delta Vpu/\Delta Nef}$ samples was calculated with respect to the SHIV$_{KU-2MC4}$ in the presence of each respective BST-2 using a Student's t-test (*). The level of infectious virus released was determined using TZM-bl indicator cells (Fig. 4B). The results are represented as the average log$_{10}$ infectious units. Significance in the restriction of infectious units released was determined with respect to the appropriate empty vector control (•) since Nef has significant effects on virion infectivity that are not associated with BST-2 incorporation (Aiken and Trono, 1995; Chowers et al., 1994). All four SHIVs exhibited similar p27 release in the absence of any BST-2 protein. In the presence of hBST-2, SHIV$_{\Delta Vpu}$ and SHIV$_{\Delta Vpu/\Delta Nef}$ viruses exhibited a significant decrease in p27 release from cells while SHIV$_{\Delta Nef}$ had no decrease in p27 release. Similar results were observed in the infectious units assay. In the presence of ptBST-2 protein, only SHIV$_{\Delta Nef}$ and SHIV$_{\Delta Vpu/\Delta Nef}$ showed a decrease in p27 release as well as infectious units released. These results are in congruence with those published by other investigators that found that hBST-2 is susceptible to HIV-1 Vpu and ptBST-2 is sensitive to the SIV Nef (Neil et al., 2008; Van Damme et al., 2008; Zhang et al., 2009), indicating that these SHIVs can serve as models for studying the individual and additive effects of different BST-2 proteins.

Mutant ptBST-2 antagonism of SHIV release

Previous studies showed that the transmembrane domain of the hBST-2 protein dictates its interaction with and susceptibility to HIV-1 Vpu (McNatt et al., 2009; Gupta et al., 2009; Rong et al., 2009). We determined if cumulative consecutive amino acid substitutions in the transmembrane domain of the ptBST-2 would result in a gradual increase in sensitivity to Vpu. We generated a library of ptBST-2 constructs with successive "humanizing" amino acid substitutions starting from the amino terminal end of the TMD with an insertion of two amino acids, a leucine and a glycine, at positions 29 and 30. We transfected each construct into 293 cells in conjunction with one of four different simian–human immunodeficiency virus expressing plasmids. The supernatants and cell lysates were used to quantify the percent p27 antigen release from cells and the supernatants were also evaluated for the number of infectious

A

```
CX54 PBMC        1 MAPILYDYRKMPMDDIWKEDGDKRCKLVVGILGLLVIVLLGVLLIFFIIKANSEACQDGLRAVMECRNVT
AH64 SPLEEN      1 --------------------------------------------------------------------
AS05 SPLEEN      1 --------C----G------------------------------P-----------------------
AS34 SPLEEN      1 --------C-----------------------------------------------------------
AS89 SPLEEN      1 --------C----G------------------------------P-----------------------
CB554098         1 --------------------------------------------------------------------
FJ943431         1 --------C----------------------I------------------------------------
FJ943432         1 --------C----G-----------------I------------------------------------
GQ304749         1 --------------------------------------------------------------------
NM_001161666     1 -------------G-----------------I------------------------------------


CX54 PBMC       71 YLLQQELAEAQRGFRDAEAQAVTCNQTVMALMASLDAEKAQGRKKVEELEGEITTLNHKLQDASAEVERL
AH64 SPLEEN     71 ---------------------------------------------------------------------
AS05 SPLEEN     71 -----------------------------------------------H---------------------
AS34 SPLEEN     71 ---------------------------------------------------------------------
AS89 SPLEEN     71 -----------------------------------------------H---------------------
CB554098        71 ---------------------------------------------------------------------
FJ943431        71 ---------------------------------------------------------------------
FJ943432        71 ---------------------------------------------------------------------
GQ304749        71 ---------------------------------------------------------------------
NM_001161666    71 ---------------------------------------------------------------------


CX54 PBMC      141 RRENHVLNARIADTDSASPQDSSCAAEPPLLILLLGLSALLL*
AH64 SPLEEN    141 ------------------------------------------*
AS05 SPLEEN    141 ------------------------------------------*
AS34 SPLEEN    141 ------------------------------------------*
AS89 SPLEEN    141 ------------------------------------------*
CB554098       141 ------------------------------------------*
FJ943431       141 ---------------G-S----------V-------------*
FJ943432       141 ------------------------------------------*
GQ304749       141 ------------------------------------------*
NM_001161666   141 -----------------S------------------------*
```

B

```
CC8X PBMC        1 MAPILYDYCKMPMDDIWKEDGDKRCKLVVGILGLLVIVLLGVLLIFFIIKANSEACQDGLRAVMECRNVT
W004 PBMC        1 --------------------------------------------------------------------
W005 PBMC        1 -----------L--------------------------------------------------------
W006 PBMC        1 --------------------------------------------------------------------
W007 PBMC        1 --------------------------------------------------------------------
W013 PBMC        1 --------------------------------------------------------------------
W018 PBMC        1 --------------------------------------------------------------------


CC8X PBMC       71 YLLQQELAEAQRGFRDAEAQAVTCNQTVMALMASLDAEKAQGRKKVEELEGEITTLNHKLQDASAEVERL
W004 PBMC       71 ---------------------------------------------------------------------
W005 PBMC       71 ---------------------------------------------------------------------
W006 PBMC       71 ---------------------------------------------------------------------
W007 PBMC       71 ---------------------------------------------------------------------
W013 PBMC       71 ---------------------------------------------------------------------
W018 PBMC       71 ---------------------------------------------------------------------


CC8X PBMC      141 RRENHVLNARIADTDSASSQDSSCAAEPPLLILLLGLSALLL*
W004 PBMC      141 ------------------------------------------*
W005 PBMC      141 ------------------------------------------*
W006 PBMC      141 --------------Y---------------------------*
W007 PBMC      141 ------------------------------------------*
W013 PBMC      141 ------------------------------------------*
W018 PBMC      141 ------------------------------------------*
```

Fig. 1. Sequence analysis of *bst-2* genes amplified from rhesus (A) and pig-tailed (B) macaques. RNA was isolated from either spleen tissue or PBMC from five rhesus macaques and seven pig-tailed macaques. The *bst-2* gene was amplified from each of these samples and bulk sequenced. The sequences obtained from the rhesus macaques are presented along with the sequences of rhBST-2 from the genomic database. The BST-2 proteins used in this study for both rhesus and pig-tailed macaques were used as references and the dashes represent similar identity.

```
Hu          1 MASTSYDYCRVPM-----EDGDKRCKLLLGIGILVLLIIVILGVPLIIFTIKANSEACRD 55
Hu LG/AA    1 .............-----.............AA........................... 55
Hu LG/II    1 .............-----.............II........................... 55
Hu ΔLG      1 .............-----.............--........................... 55
Hu T45I     1 .............-----.........................I............... 55
Hu PT-TMD   1 .............-----........V--V...G..V..L...L..F.I.......... 53

PT          1 MAPILYDYCKMPMDDIWKEDGDKRCKLV--VGILGLLVIVLLGVLLIFFIIKANSEACQD 58
PT +LG      1 ............................LG............................. 60
PT *V28L    1 ............................LLG............................ 60
PT *V31I    1 ............................LLGI........................... 60
PT *G35V    1 ............................LLGI...V....................... 60
PT *V38I    1 ............................LLGI...V..I.................... 60
PT *L41I    1 ............................LLGI...V..I..I................. 60
PT *L45P    1 ............................LLGI...V..I..I...P............. 60
PT *F48I    1 ............................LLGI...V..I..I...P..I.......... 60
PT Hu-TMD   1 ............................LLGI...V..I..I...P..I.T........ 60
PT +LG/I50T 1 ............................LG..................T......... 60
PT I50T     1 ..............................--................T......... 58
PT ΔDDIWK   1 .........-----.............--............................. 53
PT ΔDDIWK/+LG 1 .......-----..............LG............................. 55
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Fig. 2. The amino acid sequence of the N-terminal region of human and pig-tailed BST-2 proteins, and the BST-2 mutants analyzed in this study.

doses (TCID$_{50}$) (Fig. 5). The percent release of p27 in the context of each SHIV in the presence and absence of each BST-2 protein is shown in Fig. 5A. Results and significance are expressed and calculated similar to Fig. 4A. The level of infectious virus released was determined using TZM-bl indicator cells (Fig. 5B). Again, the results and significance are expressed and calculated as described for Fig. 4B. Surprisingly, a gradual increase in sensitivity to Vpu was not observed. We observed that most mutations resulted in BST-2

sensitivity to HIV-1 Vpu but the level was random in distribution. Mutations that significantly increased sensitivity to Vpu, including substitution of the leucine at position 41 with an isoleucine, did not retain this phenotype with additional "humanizing" amino acid substitutions. This indicates that the substitutions were not sufficient for the interaction with and antagonism by HIV-1 Vpu and suggests that their effect on the spatial orientation of the TMD within the membrane is also important.
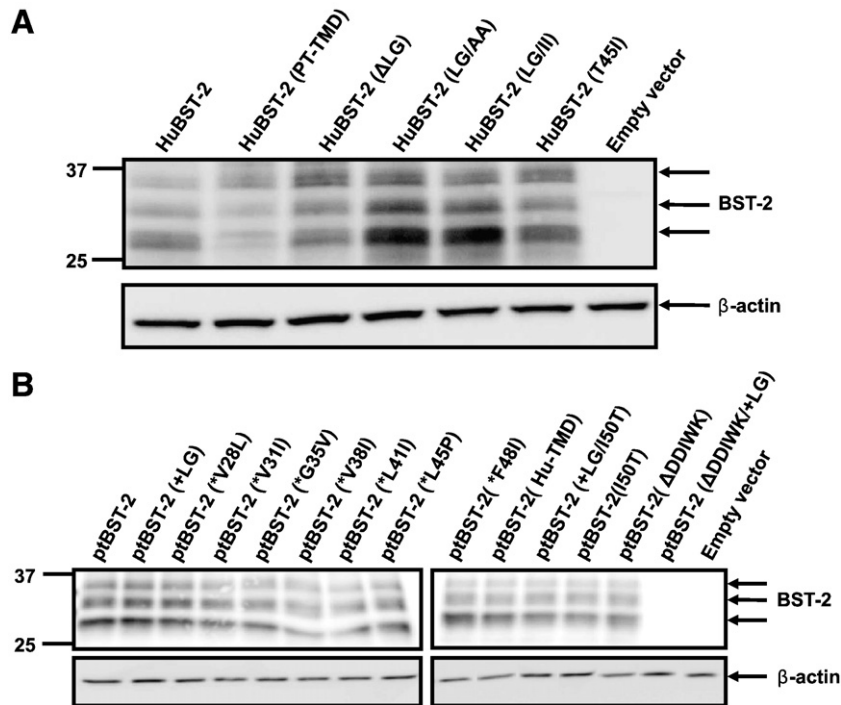

Fig. 3. Expression of parental and mutant BST-2 proteins analyzed in this study. 293 cells were transfected with vectors expressing each of the BST-2 proteins. At 48 h, cell lysates were collected and the nuclei were removed through centrifugation. The lysates were boiled in sample reducing buffer and protein expression was examined through Western blot analysis using a rabbit polyclonal anti-BST-2 antibody (NIH). Panel A. 293 cells transfected with vectors expressing human parental and mutant BST-2 proteins. Panel B. 293 cells transfected with vectors expressing pig-tailed parental and mutant BST-2 proteins.
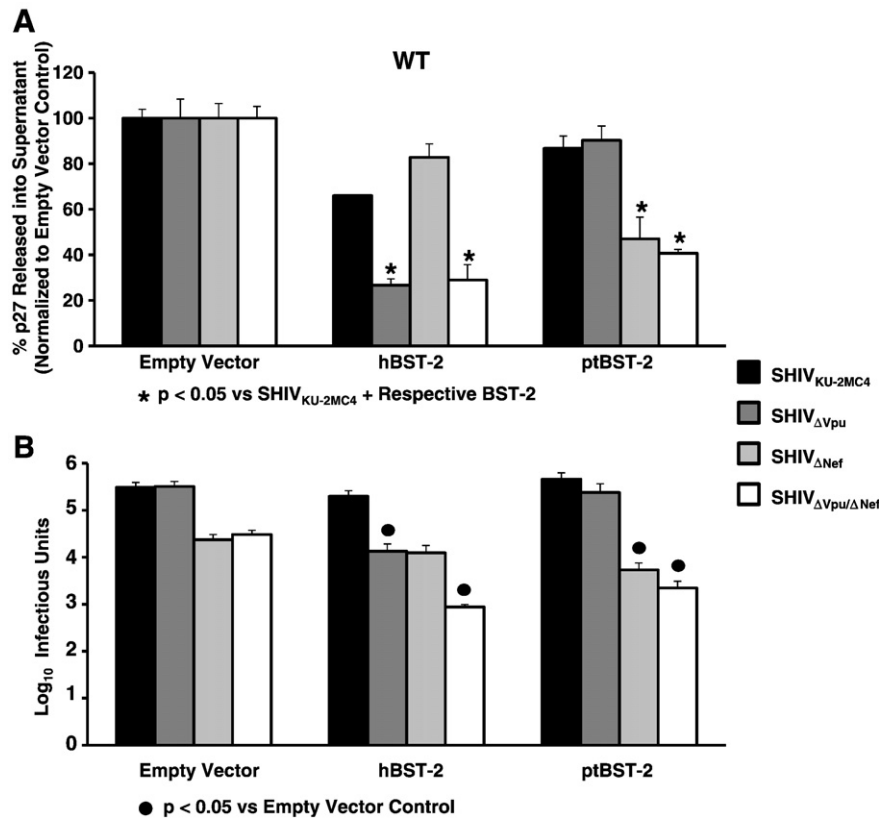
Fig. 4. BST-2 dependent down-regulation of SHIV virion release from cells transfected with hBST-2 and ptBST-2. Panel A. p27 release assay. 293 cells were co-transfected with vectors expressing proviral DNA from one of four SHIVs (SHIV$_{KU-2MC4}$, SHIV$_{\Delta Vpu}$, SHIV$_{\Delta Nef}$ or SHIV$_{\Delta Vpu/\Delta Nef}$) and a vector expressing either the hBST-2 or ptBST-2 protein. At 48 h post-transfection, the supernatants and cell lysates were collected and cleared of cellular debris and nuclei by centrifugation. The p27 content of both the supernatant and cell lysate from each sample was quantified using a p27 antigen capture assay (Zeptometrix) and the percent p27 release calculated. All conditions were run at least three separate times, normalized to their respective SHIV empty vector controls, and the average percent p27 release and standard error calculated. Significance in the restriction of p27 release for the SHIV$_{KU-2MC4}$ samples was determined with respect to the parental SHIV$_{KU-2MC4}$ empty vector control using a Student's t-test with p<0.05 considered significant (▲). Significance in the restriction of p27 release for the SHIV$_{\Delta Vpu}$, SHIV$_{\Delta Nef}$, and SHIV$_{\Delta Vpu/\Delta Nef}$ samples was calculated with respect to the SHIV$_{KU-2MC4}$ in the presence of each respective BST-2 using a Student's t-test with p<0.05 considered significant (*). Panel B. Infectious units assay using TZM-bl indicator cells. Supernatants from transfections described above were added to the TZM-bl cells and serially diluted. At 48 h post-infection, cells were washed, fixed, and stained for 2 h. The TCID$_{50}$ for each supernatant was calculated based on wells containing cells expressing β-galactosidase. All conditions were run at least three times and the TCID$_{50}$ calculated. The average TCID$_{50}$ and standard error were calculated. Significance in the restriction of infectivity was determined with respect to the parental SHIV empty vector control using a Student's t-test with p<0.05 considered significant (•).

The length of the transmembrane domain is important for hBST-2 susceptibility to Vpu

Our results shown in Fig. 5 indicated that insertion of the two residues in the transmembrane domain of ptBST-2 alone significantly increased the susceptibility to HIV-1 Vpu. These results were confirmed by quantifying the level of infectious virus released using TZM-bl indicator cells (Fig. 5B). These results suggested that the length of the transmembrane domain may be important for Vpu antagonism. To test this hypothesis, we generated additional hBST-2 constructs with different amino acid substitutions in the transmembrane domain. These constructs included hBST-2 proteins in which the TMD was replaced with the TMD from ptBST-2 (hBST-2PT-TMD), the leucine and glycine residues at positions 24 and 25 were substituted with alanines (hBST-2LG/AA), the residues at positions 24 and 25 were substituted with hydrophobic, isoleucine residues (hBST-2LG/II), or the two residues were deleted (hBST-2ΔLG). These plasmids were used in the same virion release assay described above to determine the percent virion release (Fig. 6A). The hBST-2PT-TMD protein was no longer susceptible to any viral protein, similar to results that have been published previously (McNatt et al., 2009; Douglas et al., 2009). The hBST-2ΔLG protein exhibited similar results to hBST-2PT-TMD, while the hBST-2LG/AA and hBST-2LG/II proteins produced results similar to the wild-type protein. These data suggest that alteration of the length of the transmembrane domain and not necessarily the hydrophobic nature of the amino acid residues at these

positions contributed to the resistance to HIV-1 Vpu. These results were confirmed by quantifying the levels of infectious virus released using TZM-bl indicator cells (Fig. 6B). The results and significance for both assays are expressed and calculated as described for Fig. 4.

Deletion of residues 13–17 in the ptBST-2 decreases sensitivity to SHIV Nef

In order to study the susceptibility of the ptBST-2 mutants to the SIV$_{mac}$ Nef protein encoded in our SHIVs, we generated a construct that expressed a protein with a deletion in the region previously shown to be sufficient for antagonism by Nef. Deletion of residues 13–17 within this region (ptBST-2ΔDDIWK) decreased susceptibility of the protein to SIV$_{mac}$ Nef and resulted in a decrease in p27 released from cells transfected with all four SHIV constructs (Fig. 5A). These results are in accordance with those published previously that residues 14–17 within the rhesus BST-2 protein determine susceptibility to SIV$_{mac}$ Nef. Again, these results were confirmed using TZM-bl cell assays that indicated a similar level of infectious virus (Fig. 5B).

Down-regulation of ptBST-2 mutant cell surface expression by Vpu and Nef

We analyzed the ability of Vpu to down-regulate cell surface expression of hBST-2, ptBST-2 and the ptBST-2 mutants. The results in Fig. 7 show that Vpu caused a down-regulation of hBST-2 and ptBST-2 surface expression of 47% and 14%, respectively. Replacement of the hBST-2 TMD with that of ptBST-2 protein (hBST-2PT-TMD) resulted in
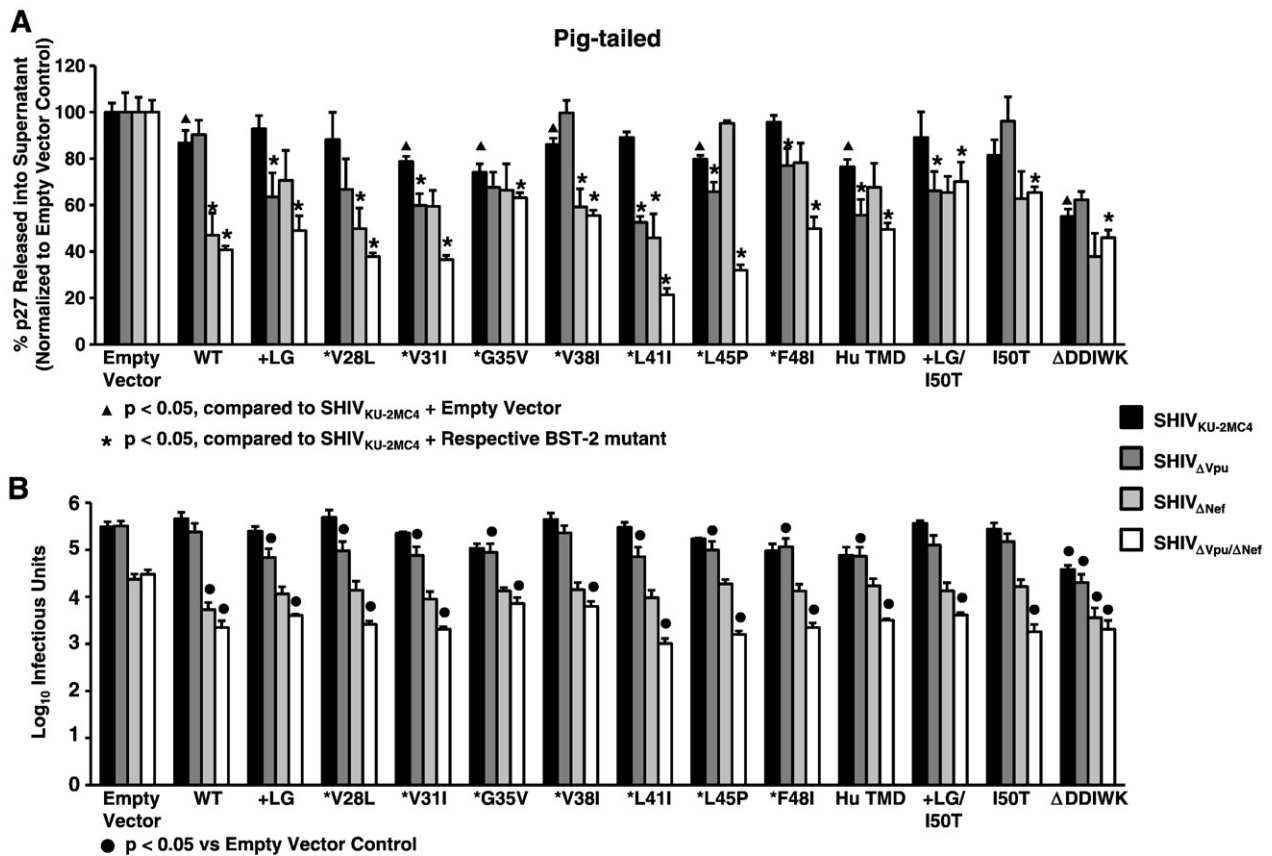
Fig. 5. BST-2 dependent down-regulation of SHIV virion release from cells transfected with vectors expressing ptBST-2 mutants. Panel A. p27 release assay. 293 cells were co-transfected with vectors expressing proviral DNA from one of four SHIVs (SHIV$_{KU-2MC4}$, SHIV$_{\Delta Vpu}$, SHIV$_{\Delta Nef}$ or SHIV$_{\Delta Vpu/\Delta Nef}$) and a vector expressing either the hBST-2 or ptBST-2 protein and a vector expressing either the parental or each mutant ptBST-2 protein. At 48 h post-transfection, the supernatants and cell lysates were collected and cleared of cellular debris and nuclei by centrifugation. The p27 content of both the supernatant and cell lysate from each sample was quantified using a p27 antigen capture assay (Zeptometrix) and the percent p27 release calculated. All conditions were run at least three separate times, normalized to their respective SHIV empty vector controls, and the average percent p27 release and standard error calculated. Significance in the restriction of p27 release for the SHIV$_{KU-2MC4}$ samples was determined with respect to the parental SHIV$_{KU-2MC4}$ empty vector control using a Student's t-test with p<0.05 considered significant (▲). Significance in the restriction of p27 release for the SHIV$_{\Delta Vpu}$, SHIV$_{\Delta Nef}$, and SHIV$_{\Delta Vpu/\Delta Nef}$ samples was calculated with respect to the SHIV$_{KU-2MC4}$ in the presence of each respective BST-2 using a Student's t-test (*). Panel B. Infectious units assay using TZM-bl indicator cells. Supernatants from transfections described above were added to the TZM-bl cells and serially diluted. At 48 h post-infection, cells were washed, fixed, and stained for 2 h. The TCID$_{50}$ for each supernatant was calculated based on wells containing cells expressing β-galactosidase. All conditions were run at least three times and the TCID$_{50}$ calculated. The average TCID$_{50}$ and standard error were calculated. Significance in the restriction of infectivity was determined with respect to the parental SHIV empty vector control using a Student's t-test with p<0.05 considered significant (•).

an increase in cell surface expression in the presence of Vpu. Our results indicate that the insertion of a leucine and glycine at positions 29 and 30 (ptBST-2 + LG) did not have a substantial effect on surface down-regulation by Vpu when compared to ptBST-2. We also did not observe an increase in Vpu-mediated surface down-regulation with *V28L and V31I mutants. However, continued humanization of the PTTMD led to a gradual increase in Vpu responsiveness. Exchange of the ptBST-2 TMD resulted in surface expression levels of 50%, similar to the hBST-2 in the presence of Vpu. We also analyzed the ability of the SHIV Nef protein to down-regulate the surface expression of ptBST-2 and hBST-2. In our hands, the SIV Nef did not significantly down-regulate the surface expression of either hBST-2 or ptBST-2 proteins (data not shown).

Discussion

Previous studies have shown that BST-2 restricts HIV-1 and SIV$_{mac}$ in a species-specific manner with these effects being counteracted by HIV-1 Vpu and SIV Nef (Jia et al., 2009; McNatt et al., 2009; Neil et al., 2008; Van Damme et al., 2008; Zhang et al., 2009). SHIVs that express both of these proteins have been used extensively to study the effects of these and other viral proteins in vivo. Thus, one goal of these studies was to determine if SHIVs would counter human and pig-tailed BST-2 proteins and if specific mutations in these primate BST-2 proteins would increase

or decrease sensitivity to Vpu and Nef. We approached this by characterizing BST-2-mediated restriction of the unmodified SHIV$_{KU-2MC4}$ and three additional SHIVs that did not express Vpu, Nef or both proteins. Our results demonstrate that SHIVs display similar properties as HIV-1 and SIV with respect to human and pig-tailed BST-2. That is, SHIV$_{\Delta Vpu}$ was restricted by hBST-2 while SHIV$_{\Delta Nef}$ was inhibited by ptBST-2. This suggests that the amino acid substitutions in the Nef protein during adaptation of SHIV$_{KU-2MC4}$ to cause disease in rhesus and pig-tailed macaques did not result in a loss of Nef antagonism against ptBST-2.

The resistance of the ptBST-2 to SHIV Vpu and its sensitivity to SHIV Nef allowed us to characterize the role of individual residues within the transmembrane domain of the ptBST-2 associated with these properties. While previous studies presented data on the role of the individual residues of the hBST-2 TMD, we used a series of sequential amino acid substitutions to "humanize" the ptBST-2 TMD (Gupta et al., 2009; McNatt et al., 2009; Rong et al., 2009). Our results are in accordance with these groups in that the two amino acid deletions in the ptBST-2 TMD (L29 and G30) are key residues involved in the susceptibility to HIV-1 Vpu. However, our analysis of a series of proteins with a progressive "humanizing" of the TMD revealed a fluctuation in sensitivity to HIV-1 Vpu. All of the ptBST-2 transmembrane domain mutants displayed susceptibility to either HIV-1 Vpu, SIV Nef or both. However, the four mutations that displayed the most interesting effects were ptBST-2(+LG), ptBST-2(*G35V), ptBST-2(*V38I) and ptBST-2(*L41I). Insertion of the leucine and glycine
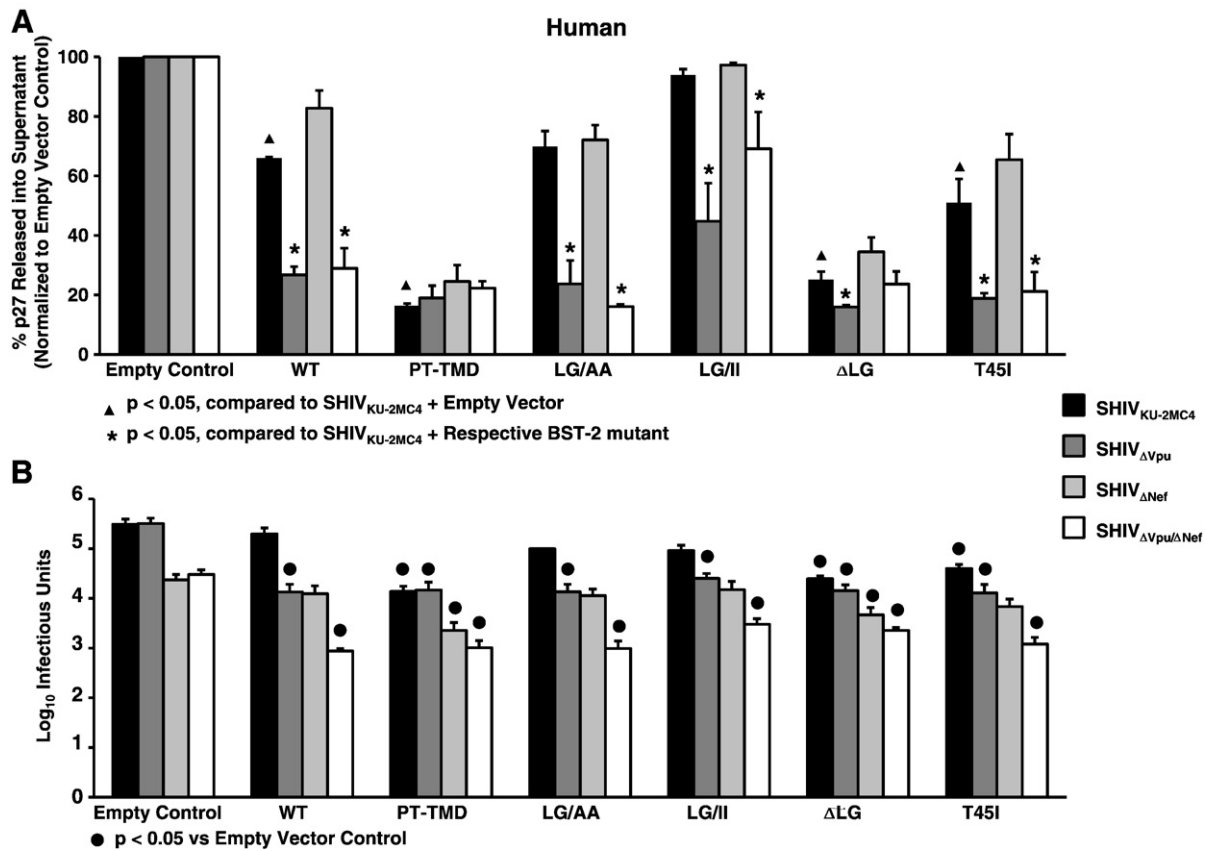
Fig. 6. BST-2 dependent down-regulation of SHIV virion release from cells transfected with vectors expressing hBST-2 mutants. Panel A. p27 release assay. 293 cells were co-transfected with vectors expressing proviral DNA from one of four SHIVs ($SHIV_{KU-2MC4}$, $SHIV_{\Delta Vpu}$, $SHIV_{\Delta Nef}$ or $SHIV_{\Delta Vpu/\Delta Nef}$) and a vector expressing either the hBST-2 or ptBST-2 protein and a vector expressing either the parental or each mutant hBST-2 protein. At 48 h post-transfection, the supernatants and cell lysates were collected and cleared of cellular debris and nuclei by centrifugation. The p27 content of both the supernatant and cell lysate from each sample was quantified using a p27 antigen capture assay (Zeptometrix) and the percent p27 release calculated. All conditions were run at least three separate times, normalized to their respective SHIV empty vector controls, and the average percent p27 release and standard error calculated. Significance in the restriction of p27 release for the $SHIV_{KU-2MC4}$ samples was determined with respect to the parental $SHIV_{KU-2MC4}$ empty vector control using a Student's t-test with $p<0.05$ considered significant (▲). Significance in the restriction of p27 release for the $SHIV_{\Delta Vpu}$, $SHIV_{\Delta Nef}$, and $SHIV_{\Delta Vpu/\Delta Nef}$ samples was calculated with respect to the $SHIV_{KU-2MC4}$ in the presence of each respective BST-2 using a Student's t-test (*). Panel B. Infectious units assay using TZM-bl indicator cells. Supernatants from transfections described above were added to the TZM-bl cells and serially diluted. At 48 h post-infection, cells were washed, fixed, and stained for 2 h. The $TCID_{50}$ for each supernatant was calculated based on wells containing cells expressing β-galactosidase. All conditions were run at least three times and the $TCID_{50}$ calculated. The average $TCID_{50}$ and standard error were calculated. Significance in the restriction of infectivity was determined with respect to the parental SHIV empty vector control using a Student's t-test with $p<0.05$ considered significant (•).
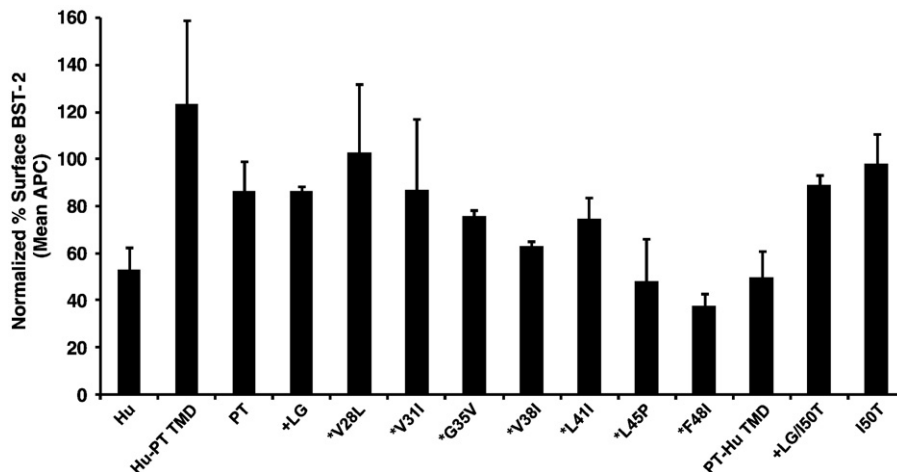

Fig. 7. The down-regulation of cell surface expression of ptBST-2 mutants by Vpu. To measure surface down-regulation of BST-2 mutants in the presence of HIV-1 Vpu, 293 cells were seeded on a 12-well format and transfected using Lipofectamine 2000. For each transfection, 0.25 μg of pCG-GFP, 0.5 μg of pVphu or mock DNA plasmid, and 0.06 μg of each BST-2 noted above were used. At 24 h, the cells were reacted with a mouse anti-HM1.24 antibody, then with an APC-conjugated goat anti-mouse antibody, followed by fixation. The cells were analyzed by two-color flow cytometry. For each BST-2 mutant, the APC mean fluorescent intensity (MFI) of high GFP expressing cells in the presence of Vpu was normalized to the MFI of cells without Vphu. The data are represented as percent BST-2 remaining on the surface of transfected cells in the presence of Vphu. All conditions were run at least twice and the standard deviation calculated.

at positions 29 and 30 clearly increased the sensitivity of the protein to HIV-1 Vpu without significantly altering the susceptibility to SIV Nef. Substitution of the glycine at position 35 with a valine eliminated the sensitivity to SHIV Vpu gained with the preceding mutations. This substitution also seemed to eliminate sensitivity to SHIV Nef. Mutation of the valine at position 38 to an isoleucine resulted in a complete loss of the sensitivity to HIV-1 Vpu gained with mutation of the preceding residues, but again without alteration of SHIV Nef susceptibility. Finally, substitution of the leucine at position 41 with an isoleucine resulted in an increased sensitivity to HIV-1 Vpu and an increase in the additive effects of Vpu and Nef. Any alterations in Nef susceptibility observed with the transmembrane domain mutations are most likely due to an alteration in the positioning of the cytoplasmic tail along the membrane. These results suggest that the overall structure, the spatial orientation of amino acids in the membrane, or possibly the tilt angle of the TMD in the membrane may be important factors. Insertion of the leucine and glycine at positions 29 and 30 into the ptBST-2 resulted in the most observable effect on the antagonism by Vpu leading us to hypothesize that the length of the transmembrane domain was important for this interaction. We tested this hypothesis by constructing three mutants of hBST-2 with either deletion of the LG (ΔLG), or substitution with amino acids with either a small hydrophobic side group (LG/AA) or a larger hydrophobic side group (LG/II). The hBST-2ΔLG deletion restricted particle release of all four SHIVs tested, while the LG/AA and LG/II mutants remained highly sensitive to Vpu and resistant to Nef. These results indicate that the length and not the degree of hydrophobicity of these amino acids is critical in defining susceptibility to HIV-1 Vpu. For these "humanized" ptBST-2 mutants, down-regulation of surface expression by HIV-1 Vpu generally correlated with the relief of restriction, with some exceptions. For example, wt ptBST-2 was minimally down-regulated by Vpu, but when ptBST-2 contained the TMD of hBST-2 it was efficiently down-regulated. Conversely, the huBST-2 mutant that contained the TMD of ptBST-2 was not down-regulated by Vpu. These results are consistent with the data on p27 release. A gradual increase in Vpu-mediated surface down-regulation was observed with sequential humanization of the ptTMD, and a reduction of ptBST-2 surface expression to levels similar to those of hBST-2 in response to Vpu was observed when the TMD was exchanged with that of hBST-2. These results generally correlated with p27 release detected with the SHIV$_{\Delta Vpu/\Delta Nef}$. However, the surface down-regulation data did not perfectly correlate with the results of p27 release. For example, the effect of Vpu on the surface expression of the ptBST(*L41I) mutant is less than expected based on the p27 release data. These results suggest that the hBST-2 transmembrane domain is involved in Vpu-mediated surface down-regulation, however it may not be sufficient to fully explain the anti-viral properties or gain in susceptibility of the ptBST-2 mutants.

The level of surface expression of hBST-2 compared to ptBST-2 is unknown and it is possible that differences in these levels may help explain the possible differences in viral restriction efficiency as well as mechanisms of antagonism by viral proteins. Differences in surface expression could be due to differences in protein expression, internalization rates and/or turnover rates of each species of protein. Studies examining these properties are necessary in order to further elucidate the mechanisms by which different species of BST-2 proteins restrict virion release and how they are antagonized by different viral proteins. Studies within our laboratory suggest that there are differences in expression levels of the hBST-2 compared to the ptBST-2 and present a possible explanation for the differences in restriction efficiency observed in this study (unpublished data). However, the studies presented herein reflect intraspecies analysis and do not indicate cross-species examination.

This study introduces SHIV as an *in vitro* model for studying BST-2 mediated restriction laying a basis for future studies that could lead to an *in vivo* model. These results also emphasize the necessity for studies focused on the structural components of BST-2 and their contributions to the anti-viral properties of the protein. These studies

support the findings by Perez-Caballero et al. (2009) that the amino acid identity of the protein is less significant than the structural properties in virion restriction. Taken together, these results provide a basis for the development of new small molecule inhibitors designed to mimic the effects of BST-2 on multiple enveloped viruses.

The physiological relevance of the anti-HIV activity of BST-2 has not yet been indicate *in vivo*. Previously published studies from our laboratory indicate that a functional Vpu protein is important for pathogenicity of SHIV in the pig-tailed macaque model, which expresses a Vpu resistant BST-2, suggesting a role for other functions of Vpu in viral pathogenesis (Hout et al., 2005; McCormick-Davis et al., 2000; Singh et al., 2001; Singh et al., 2003; Stephens et al., 2002). Of note are our studies using a SHIV expressing the VpuM2 protein (SHIV$_{M2}$). This protein has been shown to down-regulate CD4 from the surface of CD4+ cells, however it is unable to rescue virion release from cells expressing high levels of BST-2 (data not shown). SHIV$_{M2}$ caused a loss of CD4+ T cells within one month, high viral loads and histological lesions in infected macaques (Hout, et al., 2006). These studies provide evidence for additional functions of the Vpu transmembrane domain *in vivo* unrelated to BST-2 antagonism, which are potentially related to the ion channel properties exhibited by Vpu. Just as the BST-2 TM domain has amino acids/structures that are critical for interactions with Vpu, there are most likely amino acids within the Vpu TM domain that are also critical for these interactions. It will be of interest to identify these residues and determine if they are conserved in different subtypes of HIV-1.

Materials and methods

Plasmid construction

The human *bst-2* (hBST-2) gene was amplified from a plasmid expressing a full length cDNA of hBST-2 (Origene) using oligonucleotides containing 5′ BamHI and 3′ XhoI sites. The fragment was ligated into a pcDNA3.1(+) expression vector (Promega) digested with BamHI and XhoI restriction enzymes. The plasmid was sequenced to ensure no mutations were introduced during the cloning process. The pig-tailed *bst-2* (ptBST-2) and rhesus *bst-2* (rhBST-2) genes were amplified from cDNA generated from PBMC isolated from uninfected juvenile pig-tailed and rhesus macaques using a reverse-transcriptase polymerase chain reaction (RT-PCR). Oligonucleotides used contained 5′ BamHI and 3′ NotI restriction sites. The resulting fragment was ligated into a pcDNA3.1(+) expression vector (Promega) digested with BamHI and NotI restriction enzymes. Mutations introduced into all plasmids were accomplished using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. All plasmids were sequenced to ensure the validity of the mutations and that no other mutations were introduced during the cloning process. The pcVphu construct was kindly provided by the NIH AIDS Research and Reference Reagent Program.

Sequence analysis of ptBST-2 and rhBST-2 variants

The *bst-2* gene was amplified from five and seven different rhesus and pig-tailed macaque spleen tissues or PBMC, respectively, using a one-tube Titan reverse-transcriptase kit (Roche). The resulting fragments were purified using a Qiaquick Gel Extraction kit (Qiagen) and subjected to sequence analysis.

Proviral DNA plasmid construction

The construction of molecular clone SHIV$_{KU-2MC4}$ has been described previously (Liu et al., 1999). In order to construct SHIV$_{\Delta Vpu}$, SHIV$_{\Delta Nef}$, and SHIV$_{\Delta Vpu/\Delta Nef}$ another molecular clone ΔvpuΔnefSHIVPPc was used and the construction of this clone has been described previously (Joag et al., 1998). A 1444 base pair fragment was removed from SHIV$_{KU-2MC4}$

using restriction enzymes SphI and NheI. This fragment was replaced with the corresponding 1382 basepair fragment of ΔvpuΔnefSHIVPPc. The resulting construct expressed a Vpu protein with a 62 base pair deletion and an inactive start codon (SHIV$_{\Delta Vpu}$). A 525 base pair fragment was removed from SHIV$_{KU-2MC4}$ using restriction enzymes BamHI and NcoI. This fragment which encoded for part of the gp41 and nef genes was replaced with the corresponding 321 base pair fragment of ΔvpuΔnefSHIVPPc. The resulting construct expressed a nef gene with a 204 base pair deletion that included the start codon (SHIV$_{\Delta Nef}$). A 525 base pair fragment was removed from SHIV$_{\Delta Vpu}$ using restriction enzymes BamHI and NcoI. This fragment which encoded for part of the gp41 and nef genes was replaced with the corresponding 321 base pair fragment of ΔvpuΔnef SHIVPPc. The resulting construct expressed a vpu gene with a 62 base pair deletion and an inactive start codon and a nef gene with a 204 base pair deletion that included the start codon (SHIV$_{\Delta Vpu/\Delta Nef}$).

Cell lines and transfections

The HEK 293 and TZM-bl cell lines were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS), gentamicin (5 μg per ml) and penicillin/streptomycin (100U/100 μg per ml). Both cell lines were transfected using a cationic polymer (polyethylenimine) transfection reagent (ExGen™ 500, MBI Fermentas) using the manufacturer's protocol. 293 cells used for surface expression studies were maintained in Eagle's minimal essential medium (Quality Biological) supplemented with 10% FBS and 1× L-glutamine and were transfected using Lipofetamine 2000 (Invitrogen) according to manufacturer-provided instructions.

Western blot assays

Human 293 cells (10^5) were seeded into a 24-well tissue culture plate 24 h prior to transfection. Cells were transfected as described above with 1 μg of plasmid expressing full length SHIV proviral DNA (either SHIV$_{KU-2MC4}$, SHIV$_{\Delta Vpu}$, SHIV$_{\Delta Nef}$, and/or SHIV$_{\Delta Vpu/\Delta Nef}$) and empty vector or 10 ng of plasmid expressing various untagged bst-2 genes. Cells were incubated at 37 °C in 5% CO_2 atmosphere for 48 h. Cells were lysed in 200 μl of 1× RIPA buffer (50 mM Tris–HCl, pH 7.5; 50 mM NaCl; 0.5% deoxycholate; 0.2% SDS; 10 mM EDTA) and the nuclei removed through high speed centrifugation. Cell lysates were made 1× with sample reducing buffer (100 mM Tris–HCl pH 6.8, 30% glycerol, 2% SDS, 10% 2-mercaptoethanol, 0.05% bromophenol blue) and boiled for 5 min. Samples were separated on SDS-PAGE gels and transferred to PVDF membrane. BST-2 proteins were detected using a rabbit polyclonal anti-BST-2 primary antibody kindly provided by the NIH AIDS Research and Reference Reagent Program (1:1000 dilution). The secondary antibody used was an alkaline phosphatase-conjugated goat anti-rabbit IgG (whole molecule) (Sigma). Alkaline phosphatase substrate used was CDP-Star (Sigma) for chemiluminescent detection and an LAS-4000 Imager (Fujifilm) was used for the visualization and analysis of proteins.

Virion release assays

293 cells (10^5) were seeded into a 24-well tissue culture plate 24 h prior to transfection. Cells were transfected as described above with 1 μg of plasmid expressing full length SHIV proviral DNA (either SHIV$_{KU-2MC4}$, SHIV$_{\Delta Vpu}$, SHIV$_{\Delta Nef}$, or SHIV$_{\Delta Vpu/\Delta Nef}$) and 10 ng of plasmid expressing various untagged bst-2 genes. Cells were incubated at 37 °C in 5% CO_2 atmosphere for 48 h. Supernatants were collected and cellular debris removed through low speed centrifugation. Cells were lysed in 200 μl of 1× RIPA buffer (50 mM Tris–HCl, pH 7.5; 50 mM NaCl; 0.5% deoxycholate; 0.2% SDS; 10 mM EDTA) and the nuclei removed through high speed centrifugation. The amount of p27 present within the virion containing supernatant and

the cell lysates was determined using a commercially available p27 ELISA kit (Zeptometrix Incorporated) and percent p27 release calculated. All conditions were run at least three separate times, normalized to the empty vector control, and the average percent p27 release and standard error calculated. Significance in the restriction of p27 release for the SHIV$_{KU-2MC4}$ samples was determined with respect to the parental SHIV$_{KU-2MC4}$ empty vector control using a Student's t-test with $p < 0.05$ considered significant (▲). Significance in the restriction of p27 release for the SHIV$_{\Delta Vpu}$, SHIV$_{\Delta Nef}$, and SHIV$_{\Delta Vpu/\Delta Nef}$ samples was calculated with respect to the SHIV$_{KU-2MC4}$ in the presence of each respective BST-2 using a Student's t-test with $p < 0.05$ considered significant (*).

Infectious particle release assays

TZM-bl cells (10^4) expressing luciferase and β-galactosidase genes under the control of an HIV-1 promoter were seeded into a 96-well tissue culture plate 24 h prior to infection. Supernatants collected from the 293 cells co-transfected with SHIV and BST-2 expressing plasmids as described above were added to the TZM-bl cells and serially diluted. At 48 h post-infection, cells were washed twice in 1× PBS and incubated in a fixative solution (0.25% glutaraldehyde, 0.8% formaldehyde in phosphate-buffered saline) for 5 min at room temperature. The cells were washed three times in 1× PBS and covered in staining solution (400 μg/ml X-gal, 4 mM $MgCl_2$, 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6–3H_2O$ in phosphate-buffered saline) and incubated for 2 h at 37 °C. Cells were washed once in 1× PBS and then covered in 1× PBS during counting. The TCID$_{50}$ for each supernatant was calculated based on wells containing cells expressing β-galactosidase. All conditions were run at least three times and the TCID$_{50}$ calculated. The average TCID$_{50}$ and standard error were calculated. Significance in the restriction of infectivity was determined with respect to the parental SHIV empty vector control using a Student's t-test with $p < 0.05$ considered significant (•).

BST-2 surface expression assays

For surface down-regulation of BST-2 mutants in the presence of HIV-1 Vpu, 10^6 293 cells were seeded on a 12-well format and transfected using Lipofetamine 2000. For each transfection, 0.25 μg of pCG-GFP, 0.5 μg of pVphu or mock DNA plasmid, and 0.06 μg of each BST-2 noted above were used. After 24 h, the cells were stained with mouse anti-HM1.24 antibody (Chugai) at a concentration of 10 μg/ml, at 4 °C in phosphate-buffered saline (PBS) with 0.1% sodium azide and 2% fetal bovine serum, followed by APC-conjugated goat anti-mouse antibody (Biolegend) at 10 μg/ml in the same staining buffer, then fixed with 1% paraformaldehyde. The cells were analyzed by two-color flow cytometry. For each BST-2 mutant, the APC mean fluorescent intensity (MFI) of high GFP expressing cells in the presence of Vphu was normalized to the MFI of cells without Vphu. The data are represented as percent BST-2 remaining on the surface of transfected cells in the presence of Vphu. All conditions were run at least twice and the standard deviation calculated.

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