ATP1B3 Protein Modulates the Restriction of HIV-1 Production and Nuclear Factor κ Light Chain Enhancer of Activated B Cells (NF-κB) Activation by BST-2*

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Here, we identify ATP1B3 and fibrillin-1 as novel BST-2-binding proteins. ATP1B3 depletion in HeLa cells (BST-2-positive cells), but not 293T cells (BST-2-negative cells), induced the restriction of HIV-1 production in a BST-2-dependent manner. In contrast, fibrillin-1 knockdown reduced HIV-1 production in 293T and HeLa cells in a BST-2-independent manner. Moreover, NF-κB activation was enhanced by siATP1B3 treatment in HIV-1- and HIV-1ΔVpu-infected HeLa cells. In addition, ATP1B3 silencing induced high level BST-2 expression on the surface of HeLa cells. These results indicate that ATP1B3 is a co-factor that accelerates BST-2 degradation and reduces BST-2-mediated restriction of HIV-1 production and NF-κB activation.

The interferon-inducible host restriction factor BST-2/tetherin3 blocks the release of HIV-1 by directly cross-linking virions to the membranes of infected cells (1, 2). Viruses susceptible to BST-2 include all retroviruses that have been tested thus far, as well as members of the Rhabdoviridae, Paramyxoviridae, Filoviridae, and Herpesviridae families (3). Most of these viruses encode BST-2 antagonists, which degrade or remove this protein from the cell surface. The prototypical BST-2 antagonist is the HIV-1 accessory protein Vpu (1, 2). Vpu localizes primarily in endosomes and the trans-Golgi network (4, 5), where it is thought to interact with BST-2. Vpu expression results in reduced levels of BST-2 on the host cell membrane (6–8) and either the degradation (9–12) or the sequestration of the host factor in intracellular compartments (7, 13), leading to increased virus release. BST-2 has been identified as an activator of the NF-κB family of transcription factors (14), although the mechanisms by which this occurs and its consequences have remained unknown until very recently. More recently, three groups have reported that BST-2 induces NF-κB activity (15–17). These studies reported a critical role for the YXY sequence of BST-2 in signaling, which induces the canonical NF-κB pathway and suggests that BST-2 signals, at least in part, via multimerization and the nitrogen-activated protein kinase TAK1. Although the mechanisms by which BST-2 inhibits HIV-1 release are well understood, little is known regarding the host co-factor(s) of BST-2 (18–20).

Recent studies have shown that BST-2 acts as a ligand for immunoglobulin-like transcript 7 (ILT7), a receptor on plasmacytoid dendritic cells that can modulate the secretion of Toll-like receptor-mediated type I IFN and proinflammatory cytokines (21). ILT7 is a possible co-factor of BST-2 (22). However, ILT7 is a surface molecule that is selectively expressed by plasmacytoid dendritic cells (23).

In this study, we identified the binding proteins for the extra cellular domain of BST-2 using a yeast two-hybrid screen. We found that three host co-factors, epithelial cell adhesion molecule (EPCAM) (24–26), fibrillin-1 (27–29), and ATPase, Na+ /K+-transporting, β3 polypeptide (ATP1B3) (30–32), interact with BST-2. We show that the interaction of ATP1B3 with BST-2 reduces BST-2-mediated anti-HIV-1 activity.

Experimental Procedures

Construction of Plasmids—To generate the pNFLAG-BST-2 plasmid, BST-2 was amplified by RT-PCR and inserted into the BamHI and EcoRI sites of the pNFLAG-Bos plasmid (kindly provided by Dr. Takashi Suda, Kanazawa University). The pGBK7-BST-2-45–160aa was constructed by subcloning a PCR-amplified BST-2 fragment (sequence corresponding to amino acids (aa) 45–160) into the EcoRI and BamHI sites of pGBT9 (Clontech Laboratories Inc.). To construct the HA-tagged ATP1B3 and its deletion mutant expression constructs, the ATP1B3 gene and its deletion mutants were amplified by RT-PCR and inserted into the BamHI and EcoRI sites of the pcDNA3.1-HA plasmid (33). pNL4-3ΔVpu constructs were generated by using a QuikChange site-directed mutagenesis kit.

* This work was supported in part by a grant-in-aid for scientific research (C) from the Japan Society for the Promotion of Science (JSPS), Japan; by a grant-in-aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan; and by a grant from the Strategic Research Foundation grant-aided project for Private Universities from the Ministry of Education, Culture, Sport, Science, and Technology of Japan (MEXT). The authors declare that they have no conflicts of interest with the contents of this article.

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§ The abbreviations used are: BST-2/tetherin, bone marrow stromal antigen 2; EPCAM, epithelial cell adhesion molecule; ATP1B3, ATPase, Na+ /K+-transporting, β3 polypeptide; FBN1, fibrillin-1; Vpu, viral protein U; NF-κB, nuclear factor κ light chain enhancer of activated B cells; ILT7, immunoglobulin-like transcript 7; aa, amino acids.
Sodium/potassium-transporting ATP1B3 | 180–279; 228–279 | U51478.1 | 4
EPCAM | 81–202 | NM_002354.2 | 2
Fibrillin-1 | 2706–2872; 2644–2872 | NM_000138.4 | 3
E3 ubiquitin-protein ligase RING2 | 247–336 | NM_007212.3 | 2
Zinc finger protein S-arrestin | 350–532 | NM_021632.3 | 2
Keratin-like protein KRT22 | 289–347 | NM_000541.4 | 2

**TABLE 1**

**BST-2 binding proteins identified in the yeast two-hybrid screens**

| Isolated genes | Isolated fragments | Accession no. | No. of colonies |
|----------------|--------------------|---------------|----------------|
| Sodium/potassium-transporting ATP1B3 | 180–279; 228–279 | U51478.1 | 4 |
| EPCAM | 81–202 | NM_002354.2 | 2 |
| Fibrillin-1 | 2706–2872; 2644–2872 | NM_000138.4 | 3 |
| E3 ubiquitin-protein ligase RING2 | 247–336 | NM_007212.3 | 2 |
| Zinc finger protein S-arrestin | 350–532 | NM_021632.3 | 2 |
| Keratin-like protein KRT22 | 289–347 | NM_000541.4 | 2 |

* Amino acid coordinates of isolated fragments.

**Western Blotting Analysis**—The cell extracts were prepared in Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 2.5 mM β-glycerol phosphate, 1 µg/ml aprotinin, 1 µg/ml antipain, 1 µg/ml bestatin, 1 µg/ml pepstatin A, and 1 mM PMSF). The level of total protein in each sample was determined by the BCA protein assay kit (Thermo Fisher Scientific). Each sample (corresponding to 20 µg of total protein) was separated by SDS-PAGE and transferred to nitrocellulose membranes, which were blocked with 5% skim milk. The HIV-1 Gag proteins p55 and p24 were detected with the mouse monoclonal antibody 183-H12-5C (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health). The monoclonal antibodies against anti-FLAG and anti-HA antibodies were purchased from Sigma-Aldrich. A horseradish peroxidase-conjugated anti-mouse IgG antibody (Sigma-Aldrich) was used as the secondary antibody.

**FIGURE 1.** Expression analysis of BST-2, ATP1B3, fibrillin-1, and EPCAM proteins. Hela, 293, 293T, MT-4, and THP-1 cell lysates were subjected to Western blotting with the indicated antibodies.
by the chemiluminescent enzyme immunoassay (Lumipulse f, Fujirebio). The results are representative of three independent experiments, and error bars show the standard deviations of the mean values. *, p < 0.05 (Student’s t test).

Real-time RT-PCR—Total RNA was extracted from HeLa, 293T and 293 cells using an RNeasy Plus mini kit (Qiagen, Hilden, Germany), and cDNA was prepared with ReverTra Ace (Toyobo, Osaka, Japan) using oligo(dT) primers. Quantitative real-time PCR was performed with the Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), and fluorescent signals were analyzed with the StepOne RT-PCR system (Applied Biosystems). BST-2 and GAPDH genes were amplified using the following primer pairs: 5’-TCTCCTGCAACAA-GAGCTGA-3’ and 5’-CTTCTCAGTCGCTCCACCTC-3’, 5’-CGACCACCTTGTCAAGCTCA-3’ and 5’-AGGGGTCTACATGGCAACTG-3’, respectively.

Immunofluorescence—HeLa cells and siATP1B3-1 or siBST-2-treated HeLa cells were grown on polylysine-coated coverslips. The cells were fixed in 4% paraformaldehyde for 10 min at room temperature and then permeabilized in 1% Triton X-100 for 30 min at room temperature. Next, the cells were stained with the following antibodies: mouse anti-ATP1B3 (ab67409; Abcam, Burlingame, CA) and polyclonal rabbit anti-BST-2 (ab14694; Abcam), followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (Life Technologies) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Life Technologies).
respectively. Confocal microscopy analysis was performed using a Zeiss LSM 5 Pascal microscope (Carl Zeiss Microimaging, Thornwood, NY) coupled with a Kr/Ar laser (488- and 594-nm lines).

Flow Cytometric Analysis—HeLa or 293/BST-2 cells were treated with 50 nM siATP1B3-1 or 50 nM siControl for 48 h. After treatment, the cells were pretreated with 0.5% trypsin, 0.2% EDTA for 10 min to detach adherent cells and were then washed twice with PBS, 0.5% BSA. To measure cell surface protein expression, the cells were incubated with 5 μg/ml BST-2 for 30 min at 4 °C, washed again with PBS/BSA, and then stained with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life Technologies). FACS analysis was performed using the FACSCalibur and CellQuest software (BD Biosciences).

Cycloheximide Chase Assay—HeLa or 293/BST-2 cells were treated with 50 nM siATP1B3-1 or 50 nM siControl. After the cells were treated for 48 h, they were incubated with cycloheximide (100 μg/ml, Sigma-Aldrich) for the indicated times. At each time point, cells were lysed with SDS-PAGE sample buffer, and the lysates were subjected to immunoblot analysis.

Results

Identification of Binding Partners for the Extracellular Domain of BST-2—To address the roles of the extracellular domain of BST-2 in NF-κB activation and restriction of HIV-1 production, we identified the binding proteins of the extracellular domain (corresponding to aa 45–160) of BST-2 using a yeast two-hybrid screen. We performed a yeast two-hybrid screen of a human-normalized cDNA library, which had a wide range of insert sizes (generally >400 bp) that were cloned into the GAL4 activation domain plasmid pGADT7, against the plasmid pGBKT7-BST-2-45–160aa, and obtained seven interacting clones (Table 1). From these seven clones, we chose three, fibrillin-1, EPCAM, and ATP1B3, which are membrane proteins, to further characterize their roles in BST-2-mediated HIV-1 restriction and NF-κB activation.

The Depletion of ATP1B3 Reduces HIV-1 p24 Production in BST-2-expressing Cells—First, we determined the endogenous protein levels of fibrillin-1, EPCAM, and ATP1B3 expression by Western blotting analysis of HeLa, 293, 293T, MT-4, and THP-1 cells (Fig. 1). Fibrillin-1 and ATP1B3 were detected in HeLa, 293, 293T, MT-4, and THP1 cells. However, EPCAM expression was not observed in HeLa, 293, and THP-1 cells. To
address the role of fibrillin-1 and ATP1B3 in HIV-1 replication, expression of each protein was silenced by RNA interference (Fig. 2A). To eliminate any RNAi-mediated off-target effects by siRNAs, we used two or three target-specific siRNAs. HeLa cells (BST-2-positive cells), THP-1 cells (BST-2-positive macrophages), or 293T cells (BST-2-negative cells) were treated with siRNA for 24 h. After treatment, the cells were infected with vesicular stomatitis virus G pseudotyped NL4-3 (pseudo-NL4-3), NL4-3ΔVpu (pseudo-NL4-3ΔVpu), or NL4-3, NL4-3ΔVpu, corresponding to 50 ng of p24, for 48 h. Then, the levels of p24 in the supernatants were determined (Fig. 2B). When compared with control siRNA, the depletion of ATP1B3 reduced the levels of p24 in HeLa cells and THP-1 cells, but not in 293T cells, when the cells were infected with pseudo-NL4-3 or NL4-3. Moreover, this effect was also observed in cells infected with pseudo-NL4-3ΔVpu and NL4-3ΔVpu. Additionally, fibrillin-1 silencing in HeLa, 293T, and THP-1 cells led to reductions in p24 production. These results suggest that ATP1B3 plays an important role in HIV-1 replication in a BST-2-dependent manner and that fibrillin-1 supports HIV-1 replication in a BST-2-independent manner. Therefore, we focused on the role of ATP1B3 as a physiological binding partner of BST-2. Furthermore, we confirmed that off-target effects by siRNAs did not interfere with the results, and therefore, subsequent experiments were performed using siATP1B3-1.

The Extracellular Domain of ATP1B3 Binds to BST-2 at the Plasma Membrane—ATP1B3 belongs to the family of Na+/K+ and H+/K+ ATPase β chain proteins and catalyzes the hydrolysis of ATP, coupled with the exchange of Na+ and K+ ions across the plasma membrane (30–32). To evaluate whether ATP1B3 could bind to BST-2 in human cells, FLAG-BST-2 and HA-ATP1B3 were co-expressed in 293T cells. The cells were harvested for immunoprecipitation, and BST-2 was shown to bind to ATP1B3 (Fig. 3A). ATP1B3 contains a cytoplasmic domain (residues 1–35), a helical domain (residues 36–56), and an extracellular domain (residues 57–279) (31, 32). To map the binding site of ATP1B3, we constructed deletion mutants of ATP1B3 (Fig. 3B). Our results demonstrated that BST-2 efficiently co-immunoprecipitated with the full-length ATP1B3. Although the C-terminal truncation mutant Δ181–279 seemed to interact as efficiently as the wild type ATP1B3, Δ151–279 showed no detectable interaction with BST-2. To further verify the association between endogenous ATP1B3 and BST-2 in HeLa cells, we examined the intracellular localization of these two proteins using confocal microscopy and confirmed using each siRNA that ATP1B3 and BST-2 were specifically detected (Fig. 3C). Together, these results indicate that BST-2 associates with the extracellular domain of ATP1B3 at the plasma membrane.

ATP1B3 Knockdown in BST-2-expressing Cells Impairs HIV-1 Production—Next, we examined the effects of ATP1B3 on HIV-1 production in 293 and 293/BST-2 cells, which constitutively express BST-2. The BST-2 levels in 293/BST-2 cells were significantly enhanced by the depletion of ATP1B3 (Fig. 4, compare lane 5 with lane 6). Consistent with Fig. 2B, ATP1B3 knockdown in 293 cells did not affect the level of cell-associated Gag and virions. By contrast, the release efficiency of virions was modestly reduced by ATP1B3 knockdown in pseudo-NL4-3-infected 293/BST-2 cells (Fig. 4, compare lane 5 with lane 6). Moreover, this effect was enhanced in pseudo-NL4-3ΔVpu-infected 293/BST-2 cells (Fig. 4, compare lane 7 with lane 8). These results suggest that ATP1B3 suppresses the antiviral effect of BST-2.

ATP1B3 Knockdown Enhances BST-2 Expression at Cell Surfaces—To further confirm this effect, we performed flow cytometry (Fig. 5A). As expected, the levels of BST-2 were significantly higher in siATP1B3-1-transfected HeLa and 293/BST-2 cells than in siControl-transfected HeLa or 293/BST-2 cells. Notably, the level of BST-2 mRNA in siATP1B3-1-transfected cells was similar to that in siControl-transfected cells (Fig. 5B). To ascertain the stabilization of BST-2 by the depletion of ATP1B3, we performed pulse-chase analysis (Fig. 5C) and found that BST-2 was rapidly degraded in siControl-transfected HeLa or 293/BST-2 cells. In contrast, the depletion of ATP1B3 prevented the degradation of BST-2. These results indicate that ATP1B3 regulates the stability of BST-2.

ATP1B3 Knockdown Enhances BST-2-dependent NF-κB Activation—To assess the role of ATP1B3 in the activation of NF-κB by BST-2, we next performed experiments in which HeLa cells were transfected with either control or ATP1B3-specific siRNAs (siATP1B3-1) and then transfected with an

![FIGURE 4. ATP1B3 knockdown in BST-2-expressing cells impairs HIV-1 production. 293 cells stably expressing BST-2 (293/BST-2) or 293 cells were treated with 50 nM ATP1B3-1 siRNA (siATP1B3-1) or 50 nM siRNA control (siControl) for 24 h. After treatment, cells were infected with pseudo-NL4-3 or pseudo-NL4-3ΔVpu (corresponding to 50 ng of p24). At 48 h after infection, cell lysates and supernatants were analyzed by Western blotting. The values below the blots indicate the Gag signal intensities as determined by densitometry. The virus release efficiency was calculated as supernatant Gag per total Gag (cell-associated Gag plus supernatant Gag). The results are representative of three independent experiments, and error bars show the standard deviations of the mean values. *, p < 0.05 (Student’s t test).](image-url)
NF-κB reporter plasmid along with pcDNA, pNL4-3, or pNL4-3ΔVpu. Consistent with previous studies (15–17), NF-κB activation was increased by the transfection of pNL4-3 when compared with the transfection of pcDNA (Fig. 6A, 5.5-fold). The induction of NF-κB activation was higher in cells transfected with Vpu-defective pNL4-3 (11.5-fold) than in cells transfected with pNL4-3 (2.1-fold). Moreover, NF-κB activation by pNL4-3ΔVpu was enhanced by the depletion of ATP1B3 (45.8-fold). Importantly, these effects depended on BST-2 expression (Fig. 6B). In addition, the expression of BST-2-Y6,8A, which is a signaling-defective mutant, did not induce NF-κB activation. These results indicate that ATP1B3 regulates BST-2-mediated NF-κB activation. In conclusion, our findings indicate that ATP1B3 is a binding partner of BST-2 and regulates the stability of BST-2.

Discussion

BST-2 is an IFN-inducible transmembrane protein that is rapidly up-regulated on the surface of infected cells and that prevents virus release by physically tethering nascent virions to the cell membrane. However, HIV-1 overcomes this restriction factor by expressing HIV-1-Vpu, which down-regulates and degrades BST-2. Thus, although the antiviral effects of BST-2 on virus replication in infected hosts have been elucidated, little is known regarding the host co-factor(s) of BST-2 (18–20). In this study, we identified seven clones that encode proteins that bind to the extracellular domain (corresponding to aa 45–160) of BST-2 using a yeast two-hybrid screen. Finally, we chose three membrane proteins, fibrillin-1, EPCAM, and ATP1B3, to further characterize their roles in BST-2-mediated HIV-1 restriction and NF-κB activation. Although fibrillin-1 and...
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ATP1B3 were detected in HeLa, 293, 293T, MT-4, and THP-1 cells. EPCAM expression was not found in HeLa, 293T, MT-4, and THP-1 cells (Fig. 1).

To address the roles of fibrillin-1 and ATP1B3 in HIV-1 replication, each protein was silenced by RNA interference in HeLa cells (BST-2-positive cells), THP-1 cells (BST-2-positive macrophage cells), or 293T cells (BST-2-negative cells). Although ATP1B3 depletion induced the restriction of HIV-1 production in a BST-2-dependent manner, fibrillin-1 knockdown reduced HIV-1 production in a BST-2-independent manner (Fig. 2B). These results suggest that ATP1B3 plays an important role in HIV-1 replication in a BST-2-dependent manner. Therefore, we focused on the role of ATP1B3 as a physiological binding partner of BST-2. Both ATP1B3 and BST-2 were co-localized in the plasma membrane, and BST-2-associated with the extracellular domain of ATP1B3 at the plasma membrane (Fig. 3). In a previous study, a RING-type E3 ubiquitin ligase, BCA2 (breast cancer-associated gene 2; identical to Rabring7, ZNF364, or RNF115), was shown to function as a co-factor of the tetherin-interacting protein, enhancing tetherin-dependent viral restriction of HIV-1 (18, 19). More recently, BCA2 was shown to have a tetherin-independent HIV-1 antiviral activity that targets HIV-1 Gag for lysosomal degradation, impairing virus assembly and release (20). In contrast to the tetherin-dependent activity of BCA2, ATP1B3 acts antagonistically to suppress tetherin-dependent antiviral activity. In the current study, the viral envelope proteins have evolved antagonists that sequester tetherin away from the cell surface or directly induce its degradation through proteasomal or lysosomal pathways (1, 36–42). Therefore, to confirm the destabilization of BST-2 by ATP1B3, we performed pulse-chase analysis (Fig. 5C) and found that BST-2 was rapidly degraded in ATP1B3-expressing cells. In contrast, ATP1B3 depletion prevented BST-2 degradation. Therefore, ATP1B3 inhibits the anti-HIV activity of BST-2 by destabilizing the BST-2 protein.

Recent studies have shown that BST-2 serves as the ligand for ILT7, a receptor on plasmacytoid dendritic cells that negatively regulates the expression of type I interferon (21–23). Moreover, BST-2 reportedly stimulates the activity of the NF-κB family of transcription factors (15–17). Interestingly, the long isoform of tetherin was found to be an activator of NF-κB, whereas the short isoform of tetherin does not activate NF-κB, suggesting that the first 12-amino acid N-terminal sequence of the long isoform of BST2 is required for NF-κB activation (16). We also examined the influence of BST-2-mediated NF-κB induction by ATP1B3. We found that RNAi-mediated knockdown of ATP1B3 enhanced NF-κB activation in HIV-1- and HIV-1-ΔVpu-infected HeLa cells (Fig. 6A). In contrast, the non-BST-2- or mutant BST2-expressing 293 cells did not show NF-κB induction (Fig. 6B). These results suggest that ATP1B3 inhibited BST-2 activation of NF-κB due to destabilization of the BST-2 protein.

In conclusion, our findings indicate that ATP1B3 is a binding partner of BST-2 and regulates the stability of BST-2. ATP1B3 is a potential new therapeutic target for HIV and its related disorders.

Author Contributions—H. N. and H. T. conceived and designed the experiments. M. A., R. S., and H. N. performed the experiments. H. N., M. A., R. S., and H. T. analyzed the data. H. T. and H. N. wrote the paper.

Acknowledgments—We thank Klaus Strebel for providing Vpu and hBST-2 antiserum through the National Institutes of Health AIDS Research and Reference Reagent Program, Dr. T. Suda for providing pNFLAG-Bos, and Y. Taniguchi and T. Watanabe for technical assistance.

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