Vpu Directs the Degradation of the Human Immunodeficiency Virus Restriction Factor BST-2/Tetherin via a β TrCP-Dependent Mechanism[†]

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The primary roles attributed to the human immunodeficiency virus type 1 (HIV-1) Vpu protein are the degradation of the viral receptor CD4 and the enhancement of virion release. With regard to CD4 downregulation, Vpu has been shown to act as an adapter linking CD4 with the ubiquitin-proteasome machinery via interaction with the F-box protein β TrCP. To identify additional cellular β TrCP-dependent Vpu targets, we performed quantitative proteomics analyses using the plasma membrane fraction of HeLa cells expressing either wild-type Vpu or a Vpu mutant (S52N/S56N) that does not bind β TrCP. One cellular protein, BST-2 (CD317), was consistently underrepresented in the membrane proteome of cells expressing wild-type Vpu compared to the proteome of cells expressing the Vpu mutant. To verify the biological relevance of this phenotype for HIV pathogenesis, we showed that in T cells infected with HIV-1, BST-2 downregulation occurred in a Vpu-dependent manner. Recently, BST-2 has been identified as the interferon-inducible cellular factor Tetherin, which restricts HIV virion release in the absence of Vpu. We address here the unresolved mechanism of Vpu-mediated BST-2 downregulation. Our data show that the presence of wild-type Vpu reduced cell surface and total steady-state BST-2 levels, whereas that of the mutant Vpu had no effect. In addition, treatment of cells with the lysosome acidification inhibitor concanamycin A, but not treatment with the proteasome inhibitor MG132, reduced BST-2 downregulation by wild-type Vpu, thereby suggesting that the presence of Vpu leads to the degradation of BST-2 via an endosome-lysosome degradation pathway. The importance of β TrCP in this process was confirmed by demonstrating that in the absence of β TrCP, BST-2 levels were restored despite the presence of Vpu. Taken together, these data support the hypothesis that, in similarity to its role in CD4 degradation, Vpu acts as an adapter molecule linking BST-2 to the cellular ubiquitination machinery via β TrCP. However, in contrast to the proteasome-dependent degradation of CD4, which occurs in the endoplasmic reticulum, Vpu appears to interact with BST-2 in the trans-Golgi network or in early endosomes, leading to lysosomal degradation of BST-2. Via this action, Vpu could counter the tethering function of BST-2, resulting in enhanced HIV-1 virion release. Interestingly, although HIV-2 does not express Vpu, an isolate known to exhibit enhanced viral egress can downregulate surface BST-2 by an as-yet-unknown mechanism that does not appear to involve degradation. Understanding the molecular mechanisms of both Vpu-dependent and -independent antagonism of BST-2 will be critical for therapeutic strategies that exploit this novel viral function.

The human immunodeficiency virus type 1 (HIV-1) accessory protein Vpu is an 81-amino-acid (16-kDa) type I integral membrane phosphoprotein with Rev-dependent (late) expression kinetics. HIV-2 and most simian immunodeficiency virus (SIV) strains (except SIVcpz and its proposed precursors SIVgsn, SIVmus, and SIVmon) do not harbor a *vpu* gene (reviewed in reference 31), although certain HIV-2 isolates encode alternative Vpu-like functions (10, 49). While not absolutely necessary for viral replication, Vpu has several important functions that increase HIV-1 production. It has been shown that Vpu induces the degradation of CD4 (66) by targeting it to the E3 ubiquitin-ligase SCF (Skp1, Cullin, F-box)

complex (40). This interaction requires the beta transducin repeat-containing protein (β TrCP), which contains both a WD domain that interacts with Vpu and an F-box domain that interacts with the SCF ligase (3). The Vpu C terminus harbors a DSGXXS motif that, upon phosphorylation by casein kinase II (CKII), becomes a substrate for WD binding. Phosphorylation of the two serine residues within the CKII site is required for β TrCP interaction with Vpu. Previous studies indicated that Vpu is constitutively phosphorylated (55) and should therefore always be capable of interaction with β TrCP. Interestingly, it has been demonstrated that, unlike other β TrCP targets, Vpu itself is resistant to proteasomal degradation (9). This observation led to the hypothesis that Vpu sequesters β TrCP, which would consequently lead to pleiotropic defects arising from the stabilization of other β TrCP targets such as I κ B α , β -catenin, and ATF4 (6, 9). In contrast, more recent data suggest that Vpu might not be constitutively phosphorylated and that it may in fact be subject to degradation (5, 15).

The second function attributed to Vpu is the enhancement

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FIG. 1. Comparison of quantitative membrane proteomics profiles of HeLa cells expressing wild-type and mutant Vpu. (A) Schematic representation of the experimental design. HeLa-CD4 cell cultures were incubated with media containing either unlabeled amino acids or

of HIV particle release (32). Early studies aimed at mapping the regions of Vpu necessary for enhanced virus release were not as definitive as studies mapping the CD4 degradation phenotype. One group found that the phosphorylation sites in the Vpu C terminus were dispensable for virus release (17), whereas another group showed an approximately 50% reduction in virus release when these serine residues were mutated (53, 56). The importance of the transmembrane domain became apparent when it was shown that a Vpu mutant that contained a randomized transmembrane domain could still downregulate CD4 and yet failed to enhance viral release (52). The transmembrane domain has been demonstrated to confer upon Vpu the ability to either form (16, 54) or interact with (25) ion channels. These observations led to the hypothesis that Vpu modulation of the ion permeability of membranes could enhance virion release. However, no direct evidence in support of this mechanism has been found. In addition, there have been several studies suggesting that Vpu counteracts a previously unknown human host cell restriction factor that inhibits virion release (18, 51, 62). Recently, Neil et al. have shown that this factor is bone marrow stromal cell antigen 2 (BST-2) (45). They demonstrated that BST-2 is an interferon (IFN)-inducible cell surface protein that appears to "tether" HIV to the cell in the absence of Vpu, and they named the protein Tetherin to reflect this activity. Another group confirmed the tethering phenotype of BST-2 and further showed that Vpu downregulated BST-2 from the cell surface (59). Prior to these discoveries, BST-2, also known as CD317 and HM1.24, was shown to be expressed by bone marrow stromal cell lines (27), terminally differentiated B cells (22), and multiple myeloma cells (46). From a structural perspective, the protein is quite unusual, as it is a heavily glycosylated 29- to 33-kDa integral membrane protein with both a transmembrane domain and a putative glycosylphosphatidylinositol anchor (35). Intriguingly, we had previously identified BST-2 in a proteomics screening for novel targets of the viral ubiquitin ligase K5/MIR2 from Kaposi's sarcoma-associated herpesvirus, and we showed that both K5 and Vpu could downregulate BST-2 (4). Here we report that by using a similar proteomics screening for host membrane proteins that are specifically targeted by Vpu in a β TrCP-dependent manner, we consistently identified BST-2 as a major target of Vpu-mediated downregulation. Furthermore, we show that wild-type HIV-1 downregulation of BST-2 in T cells requires a Vpu with a functioning β TrCP-binding domain. Based on these results, our primary goal was to determine the molecular mechanism of Vpu-mediated downregulation of BST-2. Here, we demonstrate that

BST-2 colocalizes with both wild-type and mutant Vpu proteins in intracellular vesicular compartments that express markers of the trans-Golgi network (TGN) and early endosomes. We also show that BST-2 coimmunoprecipitates with both the wild-type and mutant Vpu proteins, the latter observation suggesting that this interaction does not require β TrCP. However, β TrCP is necessary for the subsequent Vpu-induced degradation of BST-2. Our data support a model in which, upon recycling to the TGN and early endosomes, BST-2 binds to Vpu and is subsequently targeted for lysosomal degradation in a β TrCP-dependent manner. This mechanism appears to be distinct from the Vpu- and β TrCP-dependent downregulation of CD4, in which newly synthesized CD4 in the ER is targeted for proteasomal degradation, therefore preventing CD4 from reaching the cell surface. Our work shows that Vpu induces the degradation of BST-2 in a β TrCP-dependent manner, thereby neutralizing a host strategy to restrict HIV release. In contrast, we show that an HIV-2 isolate with an enhanced viral egress phenotype has a Vpu-independent mechanism for downregulating surface BST-2.

MATERIALS AND METHODS

Cells, reagents, and antibodies. A human dermal microvascular endothelial cell (hDMVEC) line was generated as previously described (42) and maintained in human endothelial serum-free medium (Invitrogen, Carlsbad, CA) supplemented with 37 mg/ml endothelial cell growth supplement (BD, Franklin Lakes, NJ), penicillin-streptomycin-glutamine (PSG) (Invitrogen, Carlsbad, CA), and 10% human serum (Sigma, St. Louis, MO). The following cell lines were obtained from the AIDS Research & Reference Reagent Program, Division of AIDS, NIAID, NIH: CEM-green fluorescent protein (CEM-GFP) from Jacques Corbeil (19) and TZM-bl from John C. Kappes, Xiaoyun Wu, and Tranzyme Inc. (Germantown, MD) (14, 48, 58, 65). HeLa Tet-Off cells were obtained from Clontech (Mountain View, CA). All HeLa derivatives were grown in Dulbecco's modified Eagle medium supplemented with the indicated antibiotics: HeLa Tet-Off (100 mg/ml G418). CEM-GFP cells were grown in RPMI 1640 with 500 mg/ml G418. All HeLa and CEM cell lines were supplemented with 10% fetal bovine serum and PSG.

The following reagents were used at the indicated concentrations: concanamycin A (MP Biochemicals, Solon, OH) was used at 50 nM for 16 h; MG132 (Boston Biochem, Boston, MA) was used at 20 μ M for 16 h; human alpha IFN (IFN- α) (PBL Biomedical Laboratories, Piscataway, NJ) was used at 500 U/ml for 24 h; human IFN- β and IFN-1 α (PBL Biomedical Laboratories, Piscataway, NJ) were used at 500 U/ml for 24 h; human IFN- γ (PBL Biomedical Laboratories, Piscataway, NJ) was used at 10 ng/ml for 24 h; recombinant human tumor necrosis factor alpha (TNF- α) (R&D Systems, Minneapolis, MN) was used at 10 ng/ml for 24 h; and siGLO control, BST-2 SMARTpool, BTRC (β TrCP-1) SMARTpool, and FBXW11 (β TrCP-2) SMARTpool small interfering RNAs (siRNAs) (Dharmacon/Thermo Scientific, Rockford, IL) were used at 10 nM for 72 h.

The following antibodies were used for flow cytometric analysis, immunoprecipitations, and immunoblots. Mouse anti-BST-2 antibody was kindly provided by Chugai Pharmaceutical Co., Ltd. (Kanagawa, Japan) and was previously

amino acids labeled with stable heavy isotopes. These cells were then transduced with either Ad-Vpu::GFP or Ad-Vpu2/6::GFP. The cells from a pair of labeled and unlabeled cultures were then harvested, combined, and lysed. Total membranes were purified and then further fractionated. Proteins isolated from the plasma membrane fraction were then isolated, trypsinized, and analyzed by MS. (B) The values determined for proteins identified after the ASAPRatio and XPRESS filters were applied to the entire data set, and the average levels of downregulation were determined by each algorithm. (C) Representative chromatographic spectra of a BST-2 peptide (KVEELEGEEITTLNHNK) identified by both algorithms show the different normalization profiles employed and the mass spectra (MS/MS) of the peptide. (D) A flow cytometry histogram overlay depicting BST-2 expression on the x axis and percentages of maximum (max) cell numbers on the y axis. BST-2 expression levels detected using the GFP-positive population of cells, which represent HeLa-CD4 cells transduced with Ad-GFP, Ad-Vpu::GFP, or Ad-Vpu2/6::GFP, are shown. (E) HeLa Tet-Off cells were transduced with the indicated adenoviral vectors for 48 h or treated with the indicated siRNA for 72 h, and total (SDS) cell lysates were either directly subjected to SDS-PAGE and immunoblotted (IB) for BST-2 (panel 1) or first treated with PNGase F to remove added sugars (panel 2). The blot shown in panel 1 was stripped and reprobed for GFP (panel 3).

FIG. 2. BST-2 expression in HIV-1-infected T cells. (A) Flow cytometry histogram overlay depicting BST-2 expression on CEM-GFP cells infected with the indicated virus for 72 h. GFP expression in CEM-GFP cells is dependent upon HIV Tat. Therefore, the data shown here are gated on the GFP-positive, HIV-infected population. Mock, uninfected cells. (B) CEM-GFP cells were either left uninfected (Mock) or infected with the indicated virus for 72 h, and total (CHAPS) cell lysates were treated with PNGase F, subjected to SDS-PAGE, and immunoblotted (IB) for BST-2 (top panel). These blots were then stripped and reprobed for p24, GFP, and actin as indicated.

characterized (22). The mouse anti-GFP antibody clones (7.1 and 13.1) were obtained from Roche Applied Science (Indianapolis, IN), and the mouse anti-actin antibody (JLA20) was obtained from Calbiochem (Gibbstown, NJ). The anti-myc tag antibody conjugated to Alexa Fluor 647 was obtained from Cell Signaling (Danvers, MA). The following antibodies were obtained from the AIDS Research & Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1_{sf2} p24 antiserum (catalog no. 4250) and the polyclonal anti-BST-2 antibody (catalog no. 11721) were obtained from Klaus Strebel and Amy Andrew (41). The monoclonal BST-2 antibody was used for all immunoprecipitations and flow cytometry analyses as well as for the immunoblots represented in Fig. 1E (also see Fig. 8C and 9). All other immunoblot experiments utilized the polyclonal BST-2 antibody (Fig. 2B; also see Fig. 3B and 7).

Vpu and β TrCP plasmids and recombinant adenoviral vectors. The wild-type Vpu and Vpu2/6 mutant GFP fusion constructs were obtained from Richard Benarous and were previously described and characterized (6). The wild-type

β TrCP and mutant Δ F- β TrCP constructs were also kindly provided by Richard Benarous and were previously characterized (39). To avoid issues arising from poor transfection efficiencies and to ensure consistent protein expression levels, we employed a PCR for amplification of the open reading frames of the Vpu and β TrCP genes from these constructs and subcloned them into the adenoviral vector pADtet7 (2). Production of the recombinant adenovirus was performed as previously described (24). For transductions, endothelial cells were seeded at 3×10^5 cells/well in a six-well plate. The following day, adenoviral vector lysates were added to 1.2 ml of serum-free medium plus 8 mg/ml Polybrene, and spinoculation was performed by centrifugation at $400 \times g$ for 30 min. The transduced cells were then incubated at 37°C with 5% CO_2 for an additional 1.5 h followed by the addition of complete medium, and incubation was continued for 40 to 48 h. All of the adenoviral vectors contain a tetracycline-responsive promoter and therefore required the addition of the tetracycline transactivator (21), which was provided *in trans* by another adenovirus (Ad-Trans) or was constitutively expressed in HeLa Tet-Off cells. Cells were transduced at a multiplicity of infection (MOI) of 100 with both the experimental adenovirus and the tetracycline transactivator-expressing adenovirus. HeLa-CD4 cells were similarly transduced without the spinoculation step. For HeLa Tet-Off transductions, no tetracycline transactivator-expressing adenovirus was necessary, since the cells constitutively expressed the transactivator. These cells were transduced at an MOI of 20.

HIV-1 and HIV-2 production and infections. The wild-type and Vpu frame-shift mutant full-length proviral clones were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 (catalog no. 114) from Malcolm Martin (1) (referred to as HIV-1) and pNL-U₃₅ (catalog no. 968) from Klaus Strebel (57) (referred to as HIV-1 fs Δ Vpu). The HIV-1 Vpu2/6 mutation (S52N and S56N) was introduced into the pNL4-3 proviral clone by the use of standard PCR "quick change" methodologies. HIV-2 isolates were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-2_{ROD} phage from Ronald Desrosiers (43) and HIV-2_{D194}/Hut-78 from Hagen von Briesen (33, 64). All HIV viruses were subjected to titration on TZM-bl cells. CEM-GFP cells were infected at an MOI of 0.05 to 0.1 via spinoculation at $1,500 \times g$ in the presence of 10 mg/ml DEAE dextran and analyzed at 72 h postinfection.

Stable isotope labeling of amino acids in cell culture (SILAC) and sample preparation for MS analysis. The procedure for SILAC and sample preparation for mass spectrometric (MS) analysis was previously described (4). Briefly, HeLa-CD4 cells were labeled with stable nonradioactive isotopes by the use of labeling medium (Dulbecco's modified Eagle medium; Invitrogen, Carlsbad, CA) lacking the amino acids L-lysine and L-leucine (prepared according to the manufacturer's protocol). The medium was supplemented with 10% dialyzed fetal calf serum (HyClone), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen, Carlsbad, CA), and either normal L-lysine and L-leucine (Sigma, St. Louis, MO) or isotopically heavy L-lysine ($^{13}\text{C}_6$; $^{15}\text{N}_2$) and L-leucine ($^{13}\text{C}_6$; $^{15}\text{N}_1$) (Cambridge Isotope, Andover, MA). Cells were passaged for at least six doublings in labeling medium prior to initiation of the experiment to insure complete labeling.

Cells ($\sim 5 \times 10^7$) grown in labeling medium were cotransduced with Ad-Vpu: GFP (heavy labeled cells) plus Ad-Trans or Ad-Vpu2/6::GFP (light labeled cells) and Ad-Trans, each at an MOI of 100. At 40 h posttransduction, cells were harvested by scraping, washed twice in phosphate-buffered saline (PBS), resuspended in PBS containing 5 mM EDTA, and lysed by Dounce homogenization. Unlysed cells and debris were cleared from the lysate by centrifugation for 5 min at $3,000 \times g$. The cleared lysates were separated into membrane and soluble fractions by centrifugation for 30 min at $45,000 \times g$. The membrane fraction was resuspended in PBS by sonication and separated over a discontinuous sucrose gradient (2 M, 1.6 M, 1.25 M, 1.2 M, and 0.8 M) by centrifugation for 2.5 h at $100,000 \times g$. The band corresponding to the plasma membrane was isolated from the interface with 0.8 to 1.2 M sucrose, diluted fivefold in Tris-EDTA (pH 8.0), and centrifuged for 30 min at $45,000 \times g$ to pellet the proteins. The pellet was washed for 30 min in 50 mM sodium bicarbonate (pH 11.5) and centrifuged for 30 min at $45,000 \times g$, followed by a second wash in 50 mM ammonium bicarbonate (pH 8.5), and further centrifuged for 30 min at $45,000 \times g$. The final pellet was resuspended in 8.0 M deionized urea–50 mM ammonium bicarbonate (pH 8.5), and protein levels were quantified using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Samples were reduced with dithiothreitol (Sigma, St. Louis, MO) and alkylated with iodoacetamide (Sigma, St. Louis, MO) prior to overnight digestion with trypsin (Promega, Madison, WI).

Chromatography, MS, and informatics. Peptide mixtures were analyzed by electrospray ionization tandem MS (MS/MS) coupled to two-dimensional liquid chromatography, which was performed using a modified version of the protocol described by Link and coworkers (38). Briefly, 22 mg of a sample was loaded onto an Opti-Pak capillary SCX trap cartridge (Optimize Technologies, Oregon City,

OR) and eluted stepwise (12.5, 25, 37.5, 50, 62.5, 75, 87.5, 100, 112.5, 125, 200, 300, or 450 mM ammonium acetate in 0.1% formic acid) onto a reverse-phase C-18 capillary column (BioBasic-18; Thermo Electron, Waltham, MA) (180 μ m by 100 mm). Peptides were then eluted using an acetonitrile gradient (5% for 5 min, 5% to 40% for 75 min, 40% to 90% for 10 min) into a ProteomeX LCQ Deca XP Plus mass spectrometer (Thermo Electron, Waltham, MA) equipped with a low-flow (1 ml/min) electrospray source. The instrument was set to trigger data-dependent MS/MS acquisition of the three most abundant peptide ions detected during the MS survey scan when the total ion current per MS survey scan exceeded 5.0×10^5 counts. Proteins were identified by analyzing tandem mass spectra with the Sequest algorithm (Thermo Electron) as described by Yates et al. (67) by the use of the human subset of the UniProt/Swiss-Prot protein database (UniProt release 5.1; <http://www.expasy.org/sprot>). The search results were further analyzed using PeptideProphet (30). SILAC ratios were determined using both the ASAPRatio algorithm (36) and XPRESS (23). Multiple peptides derived from a single protein were included when the PeptideProphet probability value was greater than or equal to 0.85. All positive results were manually verified.

Nucleic acid isolation and real-time qPCR. RNA was isolated from hDMVECs at 48 h posttransduction using an RNeasy kit (Qiagen, Valencia, CA) with on-column DNase treatment. RNA was converted to cDNA using a Superscript III first-strand synthesis kit with priming from random hexamers (Invitrogen, Carlsbad, CA). Quantitative PCR (qPCR) was performed using Power SYBR green PCR master mix and an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Primer sequences for cDNA were as follows: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward (5'-GTCCACTGGCGTCTTCACCA-3'), GAPDH reverse (5'-GTGGCAGTGATGGCATGGAC-3'), BST-2 forward (5'-CCGCTCTGCTCGGCTTT-3'), and BST-2 reverse (5'-CCGCTCAGAACTGATGAGATCA-3'). Relative quantitation of gene expression was performed using the standard curve method as outlined by Applied Biosystems (Foster City, CA).

Flow cytometric analysis and immunofluorescence. For flow cytometric analysis of surface BST-2, cells were trypsinized and resuspended in flow cytometry buffer (1 $\times$ PBS–3% fetal bovine serum). The cells were stained for BST-2 with anti-BST-2 antibody, washed three times with flow cytometry buffer, and then stained with either an anti-mouse Alexa Fluor 647- or a Qdot 705-conjugated secondary antibody (Invitrogen), washed three times, and analyzed on a FACScalibur system (BD, Franklin Lakes, NJ). BST-2 intracellular immunofluorescence experiments were performed by first fixing the cells in 2% paraformaldehyde for 8 min, washing the cells three times in 1 $\times$ PBS, and permeabilizing the cells in 0.25% Triton X-100 for 8 min. Then, the cells were washed three times in 1 $\times$ PBS, blocked in 20% normal goat serum (NGS; Sigma, St. Louis, MO), washed once in 1 $\times$ PBS, and incubated in 1% NGS containing anti-BST-2 antibody. After primary antibody incubation, the cells were washed three times in 1 $\times$ PBS and incubated in 1% NGS with Alexa Fluor 595-conjugated secondary anti-mouse antibody (Invitrogen, Carlsbad, CA)–1 mg/ml DAPI (4',6-diamidino-2-phenylindole). For surface immunofluorescence determinations, the same protocol was followed, except the Triton X-100 step was not performed. Fluorescent images were obtained using a Leica DM IRBE confocal microscope.

Immunoblot analyses and coimmunoprecipitations. For immunoblot analyses of total cell lysates, adenoviral-vector-transduced cells were lysed in 1% sodium dodecyl sulfate (SDS) or CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) and analyzed for protein concentrations by the use of a bicinchoninic protein assay, and 10 to 50 mg of total protein in Laemmli sample buffer (10% glycerol, 5% β -mercaptoethanol, 2% SDS, 50 μ M Tris [pH 6.8], 0.02% bromophenol blue) was separated on a 4 to 12% Tris-glycine gradient gel (Fig. 1E) or a 4 to 12% NuPage gradient gel (Fig. 2B; also see Fig. 3B, 7, 8C, and 9). The proteins were transferred to polyvinylidene difluoride and immunoblotted with the mouse anti-BST-2 antibody followed by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody. The proteins were visualized using an ECL Plus or Advance chemiluminescent kit (Amersham/GE Healthcare, Pittsburgh, PA). To remove added sugars, the protein lysates were subjected to PNGase F treatment as described in the manufacturer's protocol (New England Biolabs, Ipswich, MA). To visualize the GFP fusion proteins, the blots were stripped and reprobed with an anti-GFP antibody or an anti-Vpu antibody. siRNA samples were generated by transfecting the cells with 10 nM siGLO (control) or BST-2 SMARTpool siRNA, following the Lipofectamine RNAiMax protocol (Invitrogen, Carlsbad, CA). Cells were lysed after 72 h and prepared for immunoblot analyses as described above.

For BST-2/Vpu coimmunoprecipitations, cells were first lysed in 1% CHAPS for 30 min on ice followed by centrifugation at $10,000 \times g$ for 10 min at 4°C to pellet the cellular debris. Soluble protein was then precleared with 30 μ l of a 25% protein G Plus-agarose slurry (Santa Cruz Biotechnology, Santa Cruz, CA) at

4°C for 1 h. The agarose beads were then pelleted by centrifugation at $1,000 \times g$ for 5 min at 4°C. Immunoprecipitations were performed using the supernatants by adding 1 mg of mouse anti-BST-2 antibody for 1 h at 4°C, followed by addition of 30 μ l of the 25% protein G Plus-agarose slurry and incubation for 2 h of rocking at 4°C. The immunoprecipitated complexes were then centrifuged at $1,000 \times g$ for 5 min at 4°C and washed with 500 ml of lysis buffer. The centrifugation and wash steps were repeated three times; after the final centrifugation, the supernatant was removed and the pellet was resuspended in NuPage sample buffer. The samples were boiled and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

For pulse-chase experiments, adenoviral-vector-transduced cells (6-cm-diameter dish) were first incubated in medium lacking L-methionine and L-cysteine for 30 min before being pulse-labeled for 1 h with 80 mCi/ml L-[³⁵S]methionine and 24 mCi/ml L-[³⁵S]cysteine (Express protein labeling mix; Perkin Elmer, Boston, MA). The cells were then washed three times with PBS and chased in the presence of L-methionine and L-cysteine-containing medium for the times indicated. After the chase, cells were washed with cold PBS, scraped, and lysed for 30 min in 1% CHAPS. BST-2 was immunoprecipitated with 1 mg of the antibody by the use of protein G Plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), which were then washed three times with 1% CHAPS. The proteins bound to the beads were deglycosylated by treatment with 500 U of PNGase F (New England Biolabs, Ipswich, MA), separated by SDS-PAGE, and detected by the use of both autoradiography and a Typhoon phosphorimager.

RESULTS

A proteomics screening for cellular proteins affected by Vpu identified BST-2. In an effort to identify plasma membrane proteins that are regulated by Vpu in a β TrCP-dependent manner, a comparative proteomics screening was performed using HeLa-CD4 cells, which are an HIV-susceptible, restrictive cell type. HeLa-CD4 cells were transduced with either an adenoviral vector expressing wild-type Vpu fused to GFP (Ad-Vpu::GFP) or an adenoviral vector expressing Vpu2/6 fused to GFP, which is a well-characterized Vpu mutant that cannot bind to β TrCP (Ad-Vpu2/6::GFP). These fusion proteins have been previously described (6). The Vpu2/6 protein contains two point mutations (S52N and S56N) within the C-terminal DSGXXS motif. Mutation of the two serine residues in this motif prevents the phosphorylation of Vpu by CKII, thereby eliminating Vpu binding to β TrCP. Figure 1A depicts the steps involved in the proteomics screening. The SILAC method of protein labeling was used as described previously (4) and briefly in the Materials and Methods. Plasma membrane fractions were prepared from isotopically labeled HeLa-CD4 cells that had been transduced with either Ad-Vpu::GFP or Ad-Vpu2/6::GFP. The combined fractions were analyzed by two-dimensional liquid chromatography and MS/MS. When the spectra of two independent experiments were analyzed by both the ASAPRatio and XPRESS methods, we observed that the only protein that was consistently identified by both algorithms and in each bioreplicate as exhibiting a change greater than 1.5-fold between the Vpu and Vpu2/6 samples was BST-2 (Fig. 1B). BST-2 was downregulated 2.14-fold in Vpu-expressing cells compared to Vpu2/6-expressing cells. Figure 1C shows chromatographs, mass spectra, and the sequence of one of the BST-2 peptides identified by both algorithms. To confirm the results from the proteomics screening, we obtained a highly selective monoclonal antibody directed toward human BST-2 (22) (kindly provided by Chugai Pharmaceuticals Co., Kanagawa, Japan) and performed flow cytometric analysis of either Ad-Vpu::GFP- or Ad-Vpu2/6::GFP-transduced HeLa-CD4 cells. As shown in Fig. 1D, there was a significant reduction of BST-2 on the cell surface in the

presence of wild-type Vpu. However, in the presence of Vpu2/6, there was only a modest reduction in BST-2 levels, thus independently corroborating the proteomics data and validating BST-2 as a target of Vpu. To determine whether this downregulation occurred only at the cell surface, we evaluated BST-2 protein levels in total cell SDS lysates from HeLa Tet-Off cells transduced with Ad-Vpu::GFP, Ad-Vpu2/6::GFP, or Ad-GFP. HeLa Tet-Off cells were used because they constitutively express the tetracycline transactivator, obviating the need for an additional adenoviral vector expressing the tetracycline transactivator. The use of this cell line simplified the transduction procedure and maximized the number of cells expressing the transgene. Upon immunoblotting with a BST-2-specific antibody, we observed both the putative monomeric (36-kDa) and dimeric (64-kDa) glycosylated forms of BST-2 that have previously been described by others (22, 46) (Fig. 1E, panel 1). This glycosylation was verified by treatment with PNGase F, which resulted in the predicted 17-kDa nonglycosylated form of BST-2 (Fig. 1E, panel 2). Due to the heterogeneity of the glycosylated and dimerized forms of BST-2 observed in untreated samples, PNGase F treatment was utilized for evaluating total BST-2 levels in all subsequent immunoblot experiments. All forms of BST-2 were absent from cells treated with BST-2-specific siRNAs (panels 1 and 2), thus confirming the specificity of the antibody. We observed that levels of all forms of BST-2 were significantly reduced in cells expressing Vpu compared to cells expressing either Vpu2/6 or GFP only. To verify Vpu::GFP expression in these samples, the blots shown in panels 1 and 2 were stripped and reprobed for GFP (panels 3 and 4). These data demonstrate that total cellular BST-2 levels are significantly reduced in the presence of Vpu but not in the presence of Vpu2/6.

Wild-type HIV-1 downregulates BST-2 in a Vpu- and β TrCP-dependent manner. To determine whether Vpu can downregulate BST-2 in the context of a productive HIV-1 infection, flow cytometric analysis for BST-2 was performed with HIV-1-infected T cells (CEM-GFP). As shown in Fig. 2A, wild-type HIV-1_{NL4-3} expressing a full-length functional Vpu downregulated BST-2, but a virus containing a frameshift preventing Vpu expression (HIV-1 fs Δ Vpu) had no effect on surface BST-2 levels. The HIV containing the Vpu2/6 mutation induced a slight downregulation in surface BST-2 levels.

BST-2 immunoblot analysis of these CEM-GFP cells (Fig. 2B, top panel) confirmed that only cells infected with the wild-type Vpu-expressing virus showed a marked decrease in total BST-2 levels. Interestingly, compared to uninfected CEM-GFP cell levels, the levels of BST-2 increased slightly in cells infected with HIV-1 fs Δ Vpu and HIV-1 Vpu2/6, the mutant Vpu viruses. This was likely the result of the IFN inducibility of BST-2 (data not shown). To verify equivalent infection results, the blot was stripped and reprobed for the viral capsid protein (p24) as well as for GFP, which is expressed in these cells under the control of the HIV long terminal repeat. Interestingly, although the levels of GFP, which provides a measure of infectivity, were essentially equivalent between samples, the levels of intracellular p24 varied considerably, with HIV-1 fs Δ Vpu accumulating the most p24, followed by HIV-1 Vpu2/6 and, finally, wild-type HIV-1. These data indicate that intracellular p24 accumulates more readily inside cells infected with viruses that do not downregulate BST-2,

further confirming the link between the ability of Vpu to overcome BST-2 and enhance viral release.

Overall, these data clearly demonstrate that Vpu expressed in the context of a wild-type HIV-1 infection exhibits the same BST-2 downregulation phenotype as that observed for the overexpressed Vpu::GFP fusion protein. The importance of β TrCP with respect to the ability of HIV-1 to downregulate BST-2 is further supported by the inability of HIV-1 Vpu2/6 to significantly downregulate BST-2.

BST-2 restricts virus release from HIV-1 fs Δ Vpu- and HIV-1 Vpu2/6-infected cells. To verify that BST-2 is involved in virion release, we conducted infectivity assays in the presence or absence of siRNAs directed against BST-2, which lower BST-2 protein expression below the limit of detection via immunoblot analysis (Fig. 1E, panels 1 and 2). HeLa cells were transfected with either a control siRNA or a pool of siRNAs specific for BST-2. The following day, the cells were cotransfected with a GFP-expressing control plasmid and a full-length proviral clone of HIV-1_{NL4-3}, HIV-1 fs Δ Vpu, or HIV-1 Vpu2/6. Supernatants were collected at 72 h posttransfection, and the amount of infectious viral particles released was quantitated by measuring infectivity using the TZM-bl HIV indicator cell line. As shown in Fig. 3A, when the cells were pretreated with control siRNA, HIV-1 fs Δ Vpu exhibited a 96% reduction in levels of released infectious virus compared to wild-type HIV-1. A 64% reduction was observed for HIV-1 Vpu2/6. When the cells were pretreated with BST-2 siRNA, the infectious virus yields for all constructs increased compared to control siRNA sample results. Wild-type HIV-1 levels increased 1.2-fold (120%), HIV-1 fs Δ Vpu levels increased 17-fold (71%), and HIV-1 Vpu2/6 levels increased 3.6-fold (130%). Concurrently, the intracellular p24 levels of all transfected cells exhibited a marked decrease when BST-2 was depleted by siRNA treatment (Fig. 3B). These results support the conclusions of recent studies showing that BST-2 is a potent inhibitor of HIV-1 viral release in the absence of Vpu and that this inhibition is readily circumvented in the presence of Vpu (45, 59). In addition, our data regarding HIV-1 Vpu2/6 are consistent with previous reports showing that mutations of the β TrCP-binding domain of Vpu result in partial impairment of viral release (53, 56).

Vpu downregulates TNF- α - and IFN-induced BST-2 protein but does not inhibit *bst-2* transcription induced by these cytokines. Prior to the identification of BST-2, the ability of non-permissive cells to block HIV egress was shown to be inducible by the use of IFN (44). In most primary mouse cells, BST-2 has been shown to be induced by the presence of lipopolysaccharide and both types of IFN but not by the presence of TNF- α (7). To determine whether human BST-2 was induced by similar stimuli, we first identified a cell line that did not express significant levels of BST-2. As shown in Fig. 4A, untreated immortalized hDMVECs expressed only basal levels of BST-2. However, upon treatment with IFN- α , IFN- β , and IFN- γ , BST-2 was readily detected on the cell surface. In contrast to mouse cell results, TNF- α also induced significant BST-2 expression on hDMVECs, suggesting that several antiviral and proinflammatory pathways strongly induce BST-2 transcription. Moreover, when the cells were transduced with Ad-Vpu::GFP prior to cytokine stimulation, a marked reduction in BST-2 staining was observed. These results indicate that

FIG. 3. Infectious virus release in the presence and absence of BST-2. HeLa cells were transfected with either control siRNA or a pool of BST-2-specific siRNAs for 24 h followed by transfection with proviral clones of HIV-1_{NL4-3}, HIV-1_{NL4-3} fsΔVpu, or HIV-1_{NL4-3} Vpu2/6 for 72 h. A GFP-expressing plasmid was included as a transfection control. (A) Supernatants were harvested, and released infectious virus particles were quantitated using the TZM-bl luciferase-based HIV indicator cell line. Values are given as relative light units (RLU) $\times$ 1,000. (B) The transfected cell lysates were harvested, and immunoblot analysis was performed for p24 (top blot). This blot was then stripped and reprobed for GFP (bottom blot).

Vpu can downregulate both constitutive and cytokine-inducible BST-2 levels. Because Vpu has the potential to inhibit NF- κ B by indirectly blocking the degradation of the NF- κ B inhibitor I κ B α (6, 9), we asked whether Vpu might affect the transcriptional induction of *bst-2*. qPCR was performed on RNA isolated from mock- and adenoviral vector-transduced hDMVECs stimulated with either IFN- α or TNF- α . As shown in Fig. 4B, when we compared a mock-transduced sample to mock- plus IFN- α -transduced or mock- plus TNF- α -transduced samples, we observed that the respective *bst-2* mRNA levels in IFN- α -induced and TNF- α -induced cells were approximately 20-fold and 60-fold higher than those of unstimulated cells. Transduction of these cells with either Ad-Vpu::GFP or Ad-Vpu2/6::GFP followed by IFN- α or TNF- α treatment resulted in a slightly lowered induction of the *bst-2* message (1.4 to 1.6 fold lower than that seen with the corresponding Ad-GFP-transduced cytokine-treated samples), which does not correlate with the dramatic decrease in BST-2 levels observed

for Ad-Vpu::GFP by both flow cytometric and immunoblot analysis. The *gapdh* control results indicate that none of the treatments resulted in any overall toxicity. These data suggest that Vpu does not downregulate BST-2 protein levels by affecting *bst-2* message levels. In addition, Vpu does not appear to generally inhibit the IFN- α - or TNF- α -induced responses, as *bst-2* mRNA induction by a cytokine stimulus was not significantly affected. However, the dramatic induction of *bst-2* mRNA by either cytokine was unable to counter the posttranslational inhibition of BST-2 expression by Vpu, suggesting that Vpu can efficiently downregulate the levels of BST-2 expression induced by various innate immune mechanisms.

Vpu does not inhibit synthesis of the BST-2 protein but instead increases its turnover rate. To determine whether Vpu affects the synthesis or half-life of BST-2, pulse-chase experiments were performed. HeLaTet-Off cells transduced with the Vpu-expressing adenoviral vectors were labeled with 35 S followed by various chase times in unlabeled media. Preparations for equal trichloroacetic acid-precipitable counts were immunoprecipitated with either a control antibody (immunoglobulin [Ig]) or the BST-2 antibody, and the resulting immune complexes were analyzed by SDS-PAGE and autoradiography (Fig. 5A). To determine the half-life of BST-2 for each viral vector, the BST-2-specific signal at each time point was quantitated and normalized to the zero time point (Fig. 5B). The initial amounts of BST-2 synthesized in all three treatments were equivalent, suggesting that Vpu does not overtly affect protein translation. However, the half-life of BST-2 in the presence of Vpu was notably decreased. The normal half-life of BST-2 was not reached in this assay, as the protein was stable for >24 h in the absence of Vpu (GFP control) or in the presence of Vpu2/6. In contrast, the half-life of BST-2 in Vpu-expressing cells was decreased to approximately 12 h. These data demonstrate that BST-2 is more rapidly degraded in the presence of Vpu.

Wild-type Vpu downregulates cell surface BST-2, but both wild-type Vpu and Vpu2/6 colocalize with intracellular BST-2. While the results described above suggest that Vpu acts on BST-2 in a posttranslational manner by increasing its degradation rate, they do not address the issue of where this interaction occurs. Previous studies have localized Vpu to many parts of the secretory pathway, including the ER, Golgi, TGN, and pericentriolar recirculating endosomes (11, 47, 63). Given this broad distribution, it is plausible that Vpu could interact with BST-2 and induce its degradation in several locations. For example, as has been demonstrated for CD4, Vpu could bind to nascent BST-2 in the ER and target it for cytosolic degradation via the proteasome (40). Alternatively, in similarity to the mechanism shown for several other recycling receptors (34, 37, 60), Vpu could interact with BST-2 in a post-ER compartment to redirect it to the lysosome for degradation. Since previous data indicate that BST-2 is rapidly recycled at the cell surface (50), it is conceivable that Vpu could prevent the recycling of endocytosed BST-2 back to the cell surface. Using confocal microscopy, we investigated the subcellular distribution of BST-2 in the presence of Vpu or Vpu2/6. HeLa Tet-Off cells transduced with Ad-Vpu::GFP revealed a lack of BST-2 staining on the cell surface (Fig. 6A, top panel), which is consistent with our flow cytometric data (Fig. 1D). As expected, transduction with Ad-Vpu2/6::GFP did not signifi-

FIG. 4. BST-2 mRNA and protein levels in endothelial cells stimulated with IFN/TNF- α . (A) Flow cytometry histogram overlays depicting BST-2 expression in hDMVECs treated with stimulus only, Ad-Vpu::GFP plus stimulus, or Ad-Vpu2/6::GFP plus stimulus or left untreated (Mock). hDMVECs were transduced with the indicated adenoviral vector for 24 h and then treated with the indicated stimulus for another 24 h, followed by flow cytometric analysis. (B) Graph of *bst-2* and *gapdh* mRNA levels in the presence of the indicated adenoviral vector and stimulus. hDMVECs were treated as described for panel A, total RNA was isolated from the cells, and *bst-2* and *gapdh* mRNA levels were measured via relative qPCR.

FIG. 5. BST-2 protein synthesis and turnover rate. (A) HeLa Tet-Off cells were transduced with the indicated adenoviral vectors for 40 h, pulse-labeled with [35 S]methionine for 1 h, and chased for the indicated times. Samples were then lysed in CHAPS, immunoprecipitated (IP) with either a control antibody (Ig) or the BST-2 antibody, treated with PNGase F, separated by SDS-PAGE, and detected by autoradiography. (B) Digital densitometry was performed with the image obtained from a phosphorimager, and BST-2 band intensity values were subtracted from the background value, normalized to the h 0 time point (shown as 100 on the y axis), and graphed as percent signal remaining.

cantly affect the surface staining of BST-2 (Fig. 6A, bottom panel). Interestingly, cells that expressed either Vpu or Vpu2/6 showed intracellular BST-2 colocalization to similar discrete perinuclear vesicular structures (Fig. 6B, top and middle panels). These structures appear to be located predominantly in either the TGN or recycling endosomes, as they also contained the cellular marker TGN-46 and early endosome antigen 1 (EEA-1) (see Fig. S1 in the supplemental material). In contrast, no significant colocalization was observed with the ER marker calnexin (see Fig. S1 in the supplemental material). In the absence of either Vpu or Vpu2/6, intracellular BST-2 also appeared to localize to the same perinuclear region (Fig. 6B, bottom panel). While these data suggest that Vpu does not significantly relocate intracellular BST-2, they do not rule out the possibility that Vpu retains BST-2 in an intracellular compartment and that this leads to the subsequent degradation of BST-2. While colocalization of Vpu and BST-2 has been reported previously (59), BST-2 colocalization with Vpu2/6 was unexpected, given the inability of this mutant to affect BST-2 surface levels. These data suggest that both Vpu and Vpu2/6 interact with BST-2 in an intracellular vesicular compartment but that only wild-type Vpu can target BST-2 for degradation, thereby preventing it from trafficking back to the surface.

Both wild-type and mutant Vpu directly associate with BST-2, suggesting that this interaction is independent of the presence of β TrCP. Confocal microscopy indicates that both Vpu and Vpu2/6 colocalize with BST-2 in an intracellular compartment. To determine whether this colocalization was due to a direct interaction between BST-2 and Vpu, coimmunoprecipitation experiments were performed. Lysates of HeLa Tet-Off cells transduced with Ad-Vpu::GFP, Ad-Vpu2/6::GFP, or Ad-GFP were immunoprecipitated with a BST-2-specific monoclonal antibody, and the resulting immune complexes were probed for GFP (Fig. 7, top panels). The blot was stripped and reprobed for BST-2 (middle panel). As was consistent with our immunofluorescence results, BST-2 coimmunoprecipitated with both Vpu and Vpu2/6, suggesting a direct interaction that does not involve β TrCP. We conclude from these data that the ability of Vpu to interact with BST-2 is not sufficient either to prevent BST-2 from trafficking to the cell surface or to induce BST-2 degradation.

β TrCP interaction with Vpu is necessary for BST-2 down-regulation. The phenotype of the Vpu2/6 mutant, which provided the basis for our proteomics screenings, makes it clear that Vpu serine residues S52 and S56 are involved in the downregulation of BST-2. As these two serines are necessary for Vpu to interact with β TrCP, we next investigated whether β TrCP binding to Vpu is also necessary for the downregulation of BST-2. We utilized the adenoviral vector Ad- Δ F- β TrCP, which expresses a dominant-negative form of β TrCP with the F-box domain specifically deleted. This mutant β TrCP retains the ability to interact with wild-type Vpu but can no longer bind to Skp1p ubiquitin ligase complexes (39). We reasoned that if β TrCP binding is necessary for Vpu downregulation of BST-2, then the overexpression of Δ F- β TrCP should block Vpu-induced downregulation of BST-2. Flow cytometric analysis of HeLa Tet-Off cells cotransduced with various combinations of Vpu- and β TrCP-expressing adenoviral vectors are shown in Fig. 8A. The results from the flow cytometric analysis confirm that β TrCP binding is necessary for Vpu to downregulate BST-2. When Ad- Δ F- β TrCP was cotransduced with Ad-Vpu::GFP, BST-2 surface levels returned to near wild-type levels. Conversely, overexpression of Δ F- β TrCP with Vpu2/6 had no effect on BST-2 levels (data not shown), and overexpression of wild-type β TrCP did not affect the ability of Vpu to downregulate BST-2.

To further implicate β TrCP in the Vpu-induced downregulation of BST-2, we investigated the ability of Vpu to downregulate BST-2 in the presence of β TrCP siRNA. HeLa Tet-Off cells were first transfected with a control siRNA or a pool of siRNAs specific to either β TrCP-1 or β TrCP-2 followed by transduction with the Vpu-expressing adenoviral vectors. The results of flow cytometric analysis of surface BST-2 levels shown in Fig. 8B indicate that Vpu's ability to downregulate BST-2 is inhibited when β TrCP-2 is depleted using siRNA treatment. In contrast, β TrCP-1 siRNA treatment appeared to have no effect on the Vpu-mediated downregulation of BST-2.

To verify that this BST-2 reduction occurs in total cell lysates, BST-2 immunoblot analyses were performed using these same samples. Corroborating the flow cytometry data, the immunoblot data in Fig. 8C show a marked recovery of BST-2 expression in Vpu-expressing cells treated with β TrCP-2 siRNA compared to control or β TrCP-1 siRNA results.

FIG. 6. Surface and intracellular staining of BST-2 and Vpu in HeLa Tet-Off cells. Confocal immunofluorescence images of HeLa Tet-Off cells that were transduced with the indicated virus for 40 h are shown. (A) BST-2 surface staining in which the cells were fixed and stained. Images are projections of six optical z sections. (B) Intracellular BST-2 staining images of cells were fixed, permeabilized, and then stained. Images depict a single optical z section. Green, GFP; red, BST-2; blue, DAPI.

FIG. 7. Coimmunoprecipitations of Vpu and Vpu2/6 with BST-2. HeLa Tet-Off cells were transduced with the indicated adenoviral vectors and then lysed in CHAPS. Lysates were then directly treated with PNGase F (pre-IP lysates) or first immunoprecipitated (IP) with a control antibody (IgG2a) or the BST-2 antibody (BST-2) and then treated with PNGase F followed by immunoblotting (IB) with GFP (top blots). The blots were then stripped and reprobed for BST-2 (middle blots). The pre-IP lysates represent 2.5% of the IP input and were also immunoblotted for actin as a loading control (bottom right blot). *, Ig light chain.

To determine whether the prevention of Vpu-induced degradation of BST-2 by the depletion of β TrCP-2 was functionally relevant, we performed infectious virus release assays in HeLa cells treated with β TrCP-2 siRNA as described for Fig. 3. If β TrCP-2 is required for Vpu-induced BST-2 degradation, then β TrCP-2 depletion should inhibit the viral release ability of HIV-1. As shown in Fig. 8D, cells treated with β TrCP-2 siRNA reduced HIV-1 viral release by 58%. Of note, a slight decrease in levels of released virus was also observed for HIV-1 fs Δ Vpu and HIV-1 Vpu2/6; this was likely due to the pleiotropic effects that would be expected from the depletion of an important cellular regulatory molecule.

These data clearly indicate that Vpu's interaction with β TrCP is necessary for the observed degradation of BST-2 and the consequent enhancement of virus release.

Concanamycin A, an inhibitor of the endosome-lysosome pathway, reduces Vpu-mediated degradation of BST-2. β TrCP is part of the SCF E3 ubiquitin ligase complex and is involved in ubiquitinating specific proteins, thus targeting them for degradation via the proteasome. It is in this capacity that β TrCP has been reported to be necessary for the Vpu-mediated degradation of CD4. However, β TrCP has also been shown to direct the ubiquitin-dependent endocytosis of several membrane proteins, such as the IFN- α receptor (34), the prolactin receptor (37), and the growth hormone receptor (60), which are then targeted to lysosomes for degradation. Since β TrCP is necessary for Vpu's downregulation of BST-2, we wanted to determine whether proteasomal or lysosomal pathways are involved in this process. To investigate each of these potential mechanisms, we utilized MG132, a peptide aldehyde inhibitor of the proteasome, and concanamycin A, an inhibitor of vacuolar proton ATPases that arrests vesicular transport from early to late endosomes. We predicted that MG132 and/or concanamycin A treatment would rescue the Vpu-dependent

degradation of BST-2, thereby restoring BST-2 surface expression even in the presence of wild-type Vpu. The level of total BST-2 was analyzed using HeLa Tet-Off cells that were first transduced with Ad-Vpu::GFP or Ad-Vpu2/6::GFP, followed by the addition of either MG132 or concanamycin A. Cell lysates were then treated with PNGaseF, followed by immunoblot analysis of BST-2, GFP, and cellular actin levels (Fig. 9). BST-2 in cells expressing wild-type Vpu was significantly stabilized by concanamycin A treatment, while MG132 had no effect. Concanamycin A stabilization of BST-2 in Vpu::GFP-expressing cells was evident by 3 h and reached a maximum at 8 h posttreatment. In contrast, after 14 h of concanamycin A treatment, we observed only a moderate stabilizing effect on BST-2 levels in cells expressing Vpu2/6::GFP, suggesting that the natural turnover of BST-2 was only slightly diminished in the presence of this inhibitor. Neither inhibitor had a significant effect on the levels of the Vpu fusion proteins or the cellular actin control. These data suggest that the endosome-lysosome degradation system likely plays a larger role than the ubiquitin-proteasome system in the Vpu-mediated degradation of BST-2.

HIV-2_{D194} can downregulate surface BST-2 in a Vpu-independent manner. While HIV-2 does not encode Vpu, it has been shown to downregulate CD4 via its functional Nef protein (26). Additionally, certain HIV-2 isolates have been shown to exhibit enhanced viral egress from restrictive cells (10, 49). This enhanced egress phenotype has been mapped to amino acids K422 and A598 in the extracellular domain of the viral envelope (12), with A598 having the greater impact on this phenotype (8). We therefore evaluated both an HIV-2_{ROD} egress mutant harboring substitutions at both positions (R422 and T598) and an HIV-2_{D194} isolate that maintains the A598 amino acid and is therefore predicted to exhibit enhanced viral release. Flow cytometric analyses of CEM-GFP cells that were

FIG. 8. BST-2 expression in the presence of ΔF - β TrCP or β TrCP siRNA. (A) Flow cytometry histogram overlay depicting BST-2 expression in HeLa Tet-Off cells cotransduced with Ad-GFP and Ad-control, Ad-Vpu::GFP and Ad-control, Ad-Vpu::GFP and Ad- ΔF - β TrCP, and Ad-Vpu::GFP and Ad- β TrCP for 40 h. All data were gated on the GFP-positive population; samples with β TrCP viruses were also gated on the Myc-tag-positive population, as both β TrCP constructs encode Myc-fusion proteins. (B) Flow cytometry histogram overlay depicting BST-2 expression in HeLa Tet-Off cells transfected with control, β TrCP-1, or β TrCP-2 or both β TrCP-1 and β TrCP-2 siRNAs followed by their transduction with the indicated viral vector. Cells stained with the secondary antibody only are shown as a negative control (Mock). All experimental samples were transfected for 24 h followed by adenoviral vector transduction for 40 h. (C) HeLa Tet-Off cells were treated with the

FIG. 9. BST-2 expression in the presence of Vpu and either MG132 or concanamycin A. HeLa Tet-Off cells were transduced with the indicated adenoviral vector (Ad) for 28 h followed by the addition of MG132 or concanamycin A for the specified times. Cells were then lysed in CHAPS and treated with PNGase F followed by immunoblotting (IB) with the BST-2 antibody (top blots). The top blots were then stripped and reprobed for GFP (middle blots) and then actin (bottom blots). *, glycosylated monomer form of BST-2.

infected with HIV-2_{ROD} showed no downregulation of surface BST-2 (Fig. 10A). However, the HIV_{D194} isolate downregulated BST-2 to the same degree as that observed for HIV-1. These results suggest that the enhanced viral release phenotype of HIV-2 correlates with BST-2 downregulation. To determine whether this BST-2 downregulation was caused by degradation, immunoblot analyses of total cell lysates from CEM-GFP cells infected with these two HIV-2 isolates were performed (Fig. 10B). Total BST-2 levels of both HIV-2_{ROD} and HIV-2_{D194}-infected cells actually increased compared to the mock-infected cell levels, which may reflect differences in levels of IFN induction for each isolate. These data suggest that HIV-2_{D194} overcomes BST-2 via an alternative Vpu-independent mechanism that does not appear to involve the degradation of BST-2.

DISCUSSION

Vpu's role as an adapter that links CD4 to the cellular degradation machinery via β TrCP is well established (40). Because this Vpu-mediated effect occurs posttranslationally, we reasoned that a proteomics approach would uncover novel cellular targets of Vpu that would be missed in standard microarray screenings. Surprisingly, BST-2 was the only protein identified in our screening that was consistently downregulated by wild-type Vpu but not by the Vpu2/6 mutant. The identification of BST-2 as a potential Vpu target was even more interesting, because we had previously identified the very same protein in a proteomics screening of plasma membrane proteins affected by the presence of Kaposi's sarcoma-associated herpesvirus ubiquitin ligase K5 (4). These findings suggested that BST-2 is an important target of viral immuno-

modulators. While this work was in progress, two other groups identified BST-2 as a host restriction factor that inhibits HIV virion release (45, 59) as well as the release of other retrovirus and filovirus particles (28). The first of those studies (45) focused on the function of BST-2 in virion (HIV-1 and murine leukemia virus [MLV]) release in the presence or absence of Vpu. The authors clearly showed that BST-2 inhibited both HIV-1 and MLV particle release in the absence of Vpu and that when BST-2 and Vpu were exogenously expressed, both proteins were found to colocalize within the cell. However, they were unable to demonstrate a significant effect of Vpu upon either the expression levels or subcellular distribution of BST-2; therefore, they did not directly address the mechanism of Vpu-mediated inhibition of BST-2. The second of those studies (59) provided further support for the tethering function of BST-2 and demonstrated that the presence of wild-type Vpu decreased the levels of endogenous BST-2 from the cell surface. In addition, using mutant Vpu molecules, they showed that both the transmembrane domain and the β TrCP-binding domain play a role in Vpu's ability to downregulate BST-2. While the phenotype of their transmembrane mutant may have been the result of mistargeting, the phenotype of the β TrCP-binding mutant suggested ubiquitin-proteasome involvement. However, as they were unable to prevent BST-2 downregulation with the proteasome inhibitor MG132, no definitive conclusions were made regarding the mechanism. The goal of our study was therefore to determine the mechanism of Vpu-mediated downregulation of endogenously expressed BST-2.

We first established the biological relevance of this phenotype by demonstrating that wild-type HIV-1_{NL4-3} was able to efficiently downregulate BST-2 in T cells and that this downregulation was completely dependent upon the presence of

indicated siRNA for 24 h followed by transduction with the indicated adenoviral vector for 40 h. Total (CHAPS) cell lysates were then treated with PNGase F, subjected to SDS-PAGE, and immunoblotted (IB) for BST-2 (top panel). These blots were then stripped and reprobed for GFP (bottom panel). (D) HeLa cells were transfected with either control siRNA or a pool of β TrCP-2-specific siRNAs for 24 h followed by transfection with proviral clones of HIV-1_{NL4-3}, HIV-1_{NL4-3} Δ Vpu, or HIV-1_{NL4-3} Vpu2/6 for 72 h. A GFP-expressing plasmid was included as a transfection control. Supernatants were harvested, and released infectious virus particles were quantitated using the TZM-bl Luciferase-based HIV indicator cell line. Values are provided as relative light units (RLU) $\times$ 1000.

FIG. 10. BST-2 expression in HIV-2-infected T cells. (A) Flow cytometry histogram overlay depicting BST-2 expression on CEM-GFP cells infected with the indicated HIV-2 isolates for 72 h. In CEM-GFP cells, GFP expression is dependent upon the presence of HIV Tat. The data shown here were gated on the GFP-positive, HIV-infected population. Mock, uninfected cells. (B) CEM-GFP cells were either left uninfected (Mock) or infected with the indicated virus for 72 h. Total (CHAPS) cell lysates were then treated with PNGase F, subjected to SDS-PAGE, and immunoblotted for BST-2 (top panel). These blots were then stripped and reprobed for GFP and actin.

Vpu and its interaction with β TrCP (Fig. 2). This is in contrast to a recent report suggesting that Vpu-induced degradation of BST-2 does not occur in the two T-cell lines tested (CEMx174 and H9) (41). The reason for this discrepancy is not clear but may be due to the differences in cell types and methodologies used.

In our experiments demonstrating the correlation between BST-2 downregulation and the enhanced release of HIV-1, we obtained results similar to those presented by Neil et al. (45). Decreasing BST-2 expression by siRNA treatment greatly enhanced virion release for the HIV-1 fs Δ Vpu virus but had only a minimal effect on wild-type HIV-1 release (Fig. 3). In addition, we observed an intermediate release phenotype for HIV-1 Vpu2/6, which is consistent with previous observations (53, 56). We speculate that although Vpu2/6 does not have the means to induce BST-2 degradation, it retains the ability to bind BST-2 and may therefore partially inhibit BST-2 function.

Our immunofluorescence data are consistent with Vpu and BST-2 colocalizing to the same intracellular vesicular compartment (Fig. 6B) that contains markers of both the TGN and

early endosomes (see Fig. S1 in the supplemental material). Interestingly, the Vpu2/6 mutant protein localized similarly to wild-type Vpu, and BST-2 colocalized with both proteins. We also determined that both Vpu and Vpu2/6 coimmunoprecipitated with BST-2 (Fig. 7). These data suggest that both Vpu and Vpu2/6 interact with BST-2 independently of β TrCP and that this interaction might affect the retention of BST-2 within this distinctive perinuclear region. However, as BST-2 was still readily detected on the surface of cells expressing Vpu2/6, this interaction between BST-2 and Vpu2/6 may be transient, such that BST-2 is allowed to recycle back to the cell surface.

We performed experiments that more directly addressed the role of β TrCP in BST-2 degradation. A recent publication has implicated both β TrCP forms in the Vpu-mediated downregulation of CD4 (13), and so this prompted us to evaluate the role that each of the two forms might play in BST-2 degradation. We found that only β TrCP-2 appears to be involved in Vpu's ability to downregulate BST-2 (Fig. 8B and C). We also show that depleting the cells of β TrCP-2 partially reduces the virus yield of HIV-1 (Fig. 8D), thereby reiterating its role in enhancing virus release. Interestingly, the reduction in viral release levels seen with the β TrCP-2 depletion is similar to the reduction observed for HIV-1 Vpu2/6. In both instances, Vpu can still interact with BST-2 but cannot induce degradation, suggesting that the mere interaction of Vpu with BST-2 may have some inhibitory effect. Taken together, these data definitively demonstrate that at least one form of β TrCP is necessary for the Vpu-induced downregulation of BST-2 and further suggest that the SCF ubiquitin ligase complex is required for Vpu-mediated degradation of BST-2. While it remains to be determined whether BST-2 is directly ubiquitinated upon interaction with Vpu and β TrCP, support for ubiquitin-mediated degradation was provided by subsequent experiments in which the Vpu-mediated downregulation of BST-2 was significantly inhibited by the lysosomal inhibitor concanamycin A (Fig. 9). In agreement with the results from a recent paper by Van Damme et al. (59), we observed no significant inhibition of the Vpu-induced downregulation of BST-2 when the proteasomal inhibitor MG132 was introduced. We conclude from these results that the endosome-lysosome pathway is the more likely mechanism for β TrCP-dependent, Vpu-mediated BST-2 degradation. In addition, the relatively slow rate of BST-2 turnover in the presence of Vpu ($\sim$ 12 h; Fig. 5) is more consistent with lysosomal degradation. Although β TrCP is thought to be predominantly involved in ubiquitin-proteasome-dependent degradation events, such as the Vpu-mediated degradation of CD4, there are now several examples in the literature of the β TrCP/SCF ligase controlling the ubiquitination of plasma membrane-associated targets that are then directed to the lysosome for degradation (34, 37, 60). It is therefore plausible that Vpu acts as an adapter for the ubiquitination of both CD4 and BST-2 but also that, due to their disparate locations (ER and recycling endosomes, respectively), they are subsequently degraded via different pathways. This may also explain why the authors of previous Vpu-mapping reports have concluded that CD4 degradation and virus release functions are mechanistically and spatially separable: CD4 degradation requires Vpu's C-terminal phosphorylation sites and occurs via the proteasomal ER-associated degradation pathway, whereas virus release appears to be dependent on both the transmembrane and

FIG. 11. Model of Vpu-mediated downregulation of BST-2. (A) Vpu forms a complex with BST-2 and β TrCP in the ER membrane linking BST-2 to the SCF ubiquitin ligase in a manner analogous to that seen with CD4. This interaction may lead to the polyubiquitination $[(Ub)_n]$ of BST-2, targeting it for proteasomal degradation. (B) Normal BST-2 endocytosis to the TGN and recycling to the cell surface. BST-2 may act alone in tethering virions or may instead be part of a multiprotein complex (e.g., CAML). Any of these putative complex proteins could be potential targets of Vpu. (C) Vpu forms a complex with BST-2 and β TrCP in the membrane of the TGN or recycling endosome, potentially inducing monoubiquitination (Ub) via interaction with the E3 ubiquitin SCF ligase. This would likely prevent BST-2 from recycling back to the surface and instead target it for lysosomal degradation. Vpu may or may not also be degraded in this process. Vpu2/6 forms a reversible complex with BST-2 in the TGN or recycling endosomes, but BST-2 still manages to recycle to the surface, because β TrCP cannot interact with Vpu2/6 to ubiquitinate BST-2. Although this interaction does not result in BST-2 degradation, it may partially impair BST-2 tethering function in some other manner.

C-terminal phosphorylation domains and requires Vpu localization to a post-ER compartment (53, 56). Our data clearly demonstrate that the Vpu C-terminal phosphorylation sites are important for BST-2 downregulation and that Vpu localizes to a post-ER position for the interaction with and degradation of BST-2. While we did not address the role of the Vpu transmembrane domain in BST-2 degradation, based on previous Vpu-mediated virus release data (52, 59), we hypothesize that the source of Vpu's ability to interact with BST-2 may reside in this domain.

While this work was under review, another paper involving the mechanism of Vpu-mediated downregulation of BST-2 was published (20). As is consistent with our data, the authors of that work concluded that Vpu induces the degradation of BST-2 in a β TrCP-dependent manner. However, in contrast, their data indicate that BST-2 degradation is mediated by the proteasome and not the lysosome. We believe that this discrepancy may be due to their use of an exogenously over-expressed, hemagglutinin-tagged BST-2, which may be inherently unstable compared to endogenous BST-2.

Due to HIV-1's clear dependence on Vpu for its ability to counteract BST-2, we were interested in whether HIV-2, which does not encode Vpu, could also downregulate BST-2. We found that the HIV-2_{ROD} isolate expressing the T598 substitution envelope was unable to downregulate BST-2, while the HIV-2_{D194} isolate, which contained amino acid A598 in the extracellular domain of the envelope, could indeed downregulate surface BST-2 (Fig. 10A). While cell surface BST-2 levels were reduced by the presence of HIV_{D194}, total BST-2 levels

increased somewhat (Fig. 10B), suggesting that HIV-2_{D194} counteracts BST-2 in a different manner than does HIV-1. Several possible explanations could account for the means by which HIV-2_{D194} removes BST-2 from the cell surface. For example, HIV-2 may sequester or mislocalize BST-2 inside the cell, thereby preventing its recycling to the cell surface. Alternatively, the cellular tethering machinery may be a complex of proteins that either includes or is somehow dependent upon BST-2. Conceivably, if one of the complex members were downregulated, the entire complex might then be disrupted and no longer function to block virion release. In this scenario, HIV-2 could target a member of the Tetherin complex other than BST-2. This second possibility is intriguing in light of the very recent discovery of a different cellular restriction factor affecting virion release that is referred to as calcium-modulating cyclophilin ligand (CAML) (61). This protein was identified in a yeast two-hybrid screening using Vpu as the "bait." CAML was shown to restrict both MLV and HIV-1 particle release, although the mechanism of this action is unknown. Interestingly, both Vpu and the HIV-2 envelope were able to overcome the CAML-induced restriction of virion release. Finally, an additional anti-BST-2 function has been observed for the Ebola virus glycoprotein GP, which was also found to interact with BST-2 to overcome the blockage of virus particle release. While the mechanism by which GP counteracts BST-2 is unknown, like HIV-2_{D194}, Ebola virus does not appear to induce the degradation of BST-2 (29). Further investigation into the nature of the HIV-2-mediated antagonism of BST-2 restriction is under way in our laboratory.

We propose a model (Fig. 11) in which Vpu differentially directs the degradation of BST-2. Section A of Fig. 11 depicts a proteasomal degradation pathway for BST-2 that mimics the manner in which Vpu is thought to induce the degradation of CD4 from the ER. Our data suggest that this is an unlikely pathway for Vpu-mediated degradation of BST-2. Section B illustrates the normal endocytosis and recycling of BST-2, which is the likely site of Vpu interaction with BST-2. BST-2 may also complex with other cellular proteins such as CAML that could be alternative targets of Vpu. Section C shows a pathway whereby Vpu induces the β TrCP-dependent ubiquitination of endocytosed BST-2, thereby targeting it to the lysosome for degradation. Our data suggest that this is the major pathway for Vpu-mediated degradation of BST-2.

Future studies are necessary to confirm the role of ubiquitination in the Vpu-mediated downregulation of BST-2 and to elucidate the subsequent steps required for the delivery of BST-2 to the lysosome. Determining how HIV-2 is able to overcome the host restriction of viral release without inducing the degradation of BST-2 may reveal new factors and mechanisms involved in viral immune evasion. It will also be interesting to determine whether other cellular factors such as CAML are involved in the same pathway as BST-2 or whether they are instead alternate viral release factors that have evolved either as redundant systems or as a means to counteract different viruses.

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