UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of BST-2 in host cell defense and its antagonism by HIV-1 Vpu and HIV-2 Env

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in

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by

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This thesis of Wilson Kwan is approved, and it is acceptable

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I dedicate this thesis to my loving family and to my professors at the University of California, San Diego, for their wonderful guidance and support.

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Acknowledgments

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ABSTRACT OF THESIS

The role of BST-2 in host cell defense and its antagonism by HIV-1 Vpu and HIV-2 Env

by

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Master of Science in Biology

University of California, San Diego

Professor John Guatelli, Chair

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BST-2 (tetherin, CD317) is an interferon-inducible transmembrane protein which plays an integral role in the host cell defense against lentiviruses, including HIV-1 and HIV-2. BST-2 acts by restricting the release of budding virions at the surface of infected cells. The HIV-1 accessory protein Vpu counteracts BST-2 activity by decreasing expression of the protein at the cell surface. HIV-2 also downregulates surface BST-2 levels, but uses instead an envelope glycoprotein. The activities of both HIV-1 Vpu and HIV-2 Env depend on clathrin-mediated endocytosis (CME): disruption of clathrin coat assembly via over-expression of the C-terminal domain of the clathrin assembly protein AP180 abrogated the downregulatory effect of Vpu and Env on BST-2. Although substantial data suggested that Vpu induces the ubiquitination of BST2 and blocks its

recycling to the plasma membrane, neither knockdown of Hrs, a protein involved in the sorting of ubiquitinated surface proteins to lysosomes, nor expression of mutated versions of rab11 or rab22, regulators of endosomal recycling, blocked Vpu-activity. In addition to its role in restricting nascent virions, BST-2 also serves to stimulate NF-κB activation. This activity is also inhibited by HIV-1 Vpu or HIV-2 Env. These findings suggest that HIV-1 and HIV-2 utilize specific proteins to inhibit both the restrictive and signaling activities of BST-2.

I:

Introduction

BST-2 is a type-2 integral membrane protein with a cytoplasmic N-terminus, a membrane-spanning domain, and an C-terminus modified by an GPI anchor. [6] The protein exists as a dimer associated with lipid rafts. When virions bud from the surface of infected cells, one membrane spanning domain is incorporated into the newly formed virionenvelope while the other remains embedded in the host cell membrane [1-4]. Nascent virions are thus tethered to the surface and prevented from infecting more cells.

Viral pathogens have developed an array of mechanisms to circumvent or disable components of the anti-viral host immune response. HIV-1 and HIV-2 both actively downregulate surface-levels of BST-2, enhancing the release of virus particles and potentially interfering with cellular signaling. The process by which HIV-1 and HIV-2 modulate BST-2 expression is not entirely understood. Vpu has been proposed to stimulate lysosomal and proteosomal degradation of BST-2, block recycling of BST-2 back to the surface, and trap BST-2 in intracellular membrane-bound compartments such as the *trans*-Golgi network (TGN). HIV-2 Env also traps BST-2 within intracellular membranes but does so without stimulating its degradation [25,26].

To determine the role of the endocytic pathway in the counteraction of BST-2, we sought various methods to perturb clathrin-mediated endocytosis (CME). CME was implicated in part because HIV Env contains a clathrin adaptor protein (AP) complex binding motif which is crucial for HIV-2 Env mediated removal of surface BST-2. The primary target of modulation in my experiments was the clathrin assembly protein AP180, which is required for the assembly of clathrin lattices on membranes. S collaborating laboratory member, David Lau, utilized dynamin 2 (Dyn2, pinchase).

Dynamin 2 is a GTPase which aids in membrane scission during the formation of clathrin-coated and non-clathrin coated vesicles. Expressing a dominant-negative form of the protein inhibited the down-regulatory activities of HIV-1 Vpu and HIV-2 Env. Over-expression of the C-terminal fragment of AP-180 had a similar effect. Immunofluorescence microscopy ensured that these perturbations had no adverse effect on the sub-cellular distribution of Vpu or Env. Neither Vpu nor Env affected the rate of BST-2 endocytosis; this led us to propose that decreased surface levels of BST-2 result from a block to recycling of the protein.

Further exploration of this concept was performed by perturbation of several steps along the post-endocytic pathway. Knocking down expression of Hrs, an adaptor protein which targets endosomes to lysosomes, had no significant impact on Vpu activity. Likewise, expression of dominant-negative Rab-11 and Rab-22 (GTPases implicated in the trafficking of recycling endosomes) showed no effect on the ability of Vpu to downregulateBST-2. Thus, the precise point at which recycling is blocked remains undefined.

In addition to restricting budding viruses, BST-2 also stimulates the production of NF-kB, a broad regulator of transcriptional activity. NF-κB modulates both the host immune response as well as the expression viral genes and has been proposed to play a role in viral transcription [7]. I observed that HIV-1 Vpu and HIV-2 Env both inhibit BST-2-induced NF-κB activation. The Vpu effect on NF-κB activation is dependent upon residues that enable it to bind to βTRCP, a substrate adaptor for a ubiquitin ligase complex. Interestingly, expression of Env proteins from HIV-2 isolates defective for

enhancement of viral release by counteracting BST-2 (ROD14, ROD10-Y707A) still showed robust inhibition of BST-2-mediated NF-κB activation. Whether the modulation of BST-2 by Vpu and Env reflects the need of viruses to counteract restricted release or the activation of NF-κB, or both, is not yet clear.

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II:

Materials and Methods

Plasmids, reagents and antibodies

pcDNA3.1 (Invitrogen), pCA2 (a pcDNA-based plasmid expressing HLA-A2 provided by Olivier Schwartz), pCIneo (Promega) or pCDM8 were used as empty vector controls or a control expressing an irrelevant protein. pcDNA-BST2 was mutated to encode BST2-Y6,8A using the QuickChange kit (Stratagene) and confirmed by nucleotide sequencing. Dynamin 2-encoding plasmids, DN(K44A)-Dyn2 and WT-Dyn2 (His/Myc- and GFP-tagged versions) were obtained from Mark McNiven. pVphu, encoding a codon-optimized Vpu independent of Rev, was provided by Klaus Strebel. A plasmid expressing HIV-2 ROD-10 Env was provided by Paula Cannon [8]. HIV-2 Env expression plasmids from isolates ROD10 and ROD14, and the ROD10($Y_{707}A$) mutant, have been previously described [8,9]. The pcDNA3.1-based and codon-optimized Vpu2/6 and Vpu AAA/FFF mutants was constructed previously [12]. A plasmid expressing HIV-1 Rev was cloned in our laboratory previously. A plasmid expressing GFP (pcgGFP) was provided by Jacek Skowronski. A plasmid expressing Tac (IL-2 receptor α chain)-DKQTLL was provided by Juan Bonifacino [10]. A plasmid expressing AP 180-C fused to GFP was provided by Massimo Pizzato [11]. A plasmid encoding the reporter pmCyan was obtained from Invitrogen. Fluorophore labeled transferrin and EGF were obtained from Molecular Probes/Invitrogen. Rabbit antibody to HIV-2ST gp120 was obtained from the AIDS Research and Reference Reagent Program and contributed by Raymond Sweet, SmithKline Beecham Pharmaceuticals. The murine monoclonal antibody against BST-2/HM1.24 used for most cell surface staining was a gift from Chugai Pharmaceutical Co (Kanagawa, Japan). For the surface deposition assays unconjugated

and phycoerythrin (PE)-conjugated and BST-2 clone RS38 was obtained from BioLegend, San Diego. Unconjugated anti-Hrs antibodies were obtained from Bethyl Laboratories. The pGL4.32 plasmid vector encoding an NF-kB response element was obtained from Promega. shRNA constructs targeting Hrs were expressed from the vector pGFP-V-RS and obtained from OriGene. Wild-type and dominant-negative constructs of Rab-11 (S25N) and Rab-22 (S19N, Q64L) were provided by Javier Magadan. Rab-11 wild-type was mutated to Q70L using the QuickChange kit (Stratagene) and confirmed by nucleotide sequencing.

Cells and transfections

HeLa cells are the clone P4R5 (except where noted), a stable line expressing CD4 and CCR5, and were obtained from Nathaniel Landau and maintained in DMEM supplemented with 10% PBS, penicillin/ streptomycin and puromycin. HEK293T cells were also obtained from Nathaniel Landau and maintained in EMEM supplemented with 10% PBS, and penicillin/streptomycin. HeLa Z24 cells, a stable line expressing CD4 and CCR5 though lacking puromycin resistance were obtained from Chris Aiken and maintained in 10% PBS with penicillin/ streptomycin. For most experiments, cells were plated in wells of 12-well plates and transfected with 1.6 µg of total DNA using Lipofectamine 2000 (Invitrogen) and manufacturer-recommended procedures. For immunofluorescence, cells were plated in 24-well plates and transfected with 0.8 µg of total DNA. Where indicated, cells were plated in 6-well plates and transfected with 2.5 µg of total DNA or 10 cm dishes and transfected with 24 µg of total DNA. For luciferase reporter assays, cells were plated in 96-well plates and transfected with 0.2 µg of total

DNA. The amounts of specific plasmids used are indicated in the figure legends. Assays were performed approximately 16-24 hours post-transfection, unless otherwise indicated.

Western blotting

Cells were detached from plates using 0.5 mM EDTA in PBS, pelleted and resuspended in Western loading buffer containing SDS and DTT. Lysates were boiled and loaded onto 12% polyacrylamide gel for SDS-PAGE, blotted onto PVDF membranes, and analyzed as follows. His-Myc-tagged dynamin 2, p55 Gag, Vpu, HIV-2 Env, and actin were probed using horseradish peroxidase (HRP)-conjugated mouse anti-His (Sigma), mouse anti-p24 (clone 31-90-25 from ATCC), rabbit anti-Vpu (from the AIDS Research and Reference Reagent Program and contributed by Klaus Strebel), rabbit anti-Env (described above), and anti-actin (Sigma). p55, Vpu, HIV-2 Env, and actin were detected with HRP-conjugated secondary antibodies: goat anti-mouse IgG and goat anti-rabbit IgG (both from Biorad). The blot was treated with chemi-luminescent HRP substrate (GE Amersham) and visualized using auto-radiographic film (Danville Scientific).

Flow cytometry

Cells were detached from plates with 0.25% Trypsin/EDTA. Incubations with antibodies were done at 4°C. An indirect method was used to stain the cell surface for BST-2: cells were treated with a primary mouse anti-BST2 (Chugai) at 10 µg/ml, followed by an allophycocyanin (APC)-conjugated secondary goat anti-Mouse IgG (BioLegend). For Tac_DKQTLL, a direct stain was done using phycoerythrin (PE)-

conjugated antibody to CD25 (Becton Dickinson). For surface deposition assays, cells were treated with anti-BST2 clone RS38 at 4°C to block antigenic sites, then incubated at 37°C for various times before restaining with PE-conjugated RS38. The cells were washed using PBS/sodium azide/2%. After staining, all cells were fixed in 1% formaldehyde/PBS and analyzed using two-color flow cytometry. Flow cytometric gates were set using cells stained with mouse isotype-matched IgG antibodies, or in the case of GFP by using cells not expressing these fluorescent proteins. Composite data profiles were created using the FlowJo application package (TreeStarInc).

shRNA knockdowns

HeLa Z24 cells were transfected with anti-HRS shRNA in the vector pGFP-V-RS, which also encodes for puromycin resistance. Cells were initially transfected with the shRNA constructs and allowed to grow in puromycin-free media for 24 hours before the media was replaced with a media consisting of DMEM supplemented with 10% PBS, penicillin/ streptomycin and 2 µg /ml puromycin. This allowed for selection of cells which successfully expressed the transfected vector. Following a 48-hour selection period, the cells were transfected with either Vpu or an empty-vector plasmid and an additional marker (pmCyan). Cells were collected 24 hours later and analyzed by flow-cytometry.

Immunofluorescence microscopy

Cells were fixed in 3% formaldehyde/PBS followed by permeabilization in 0.1% NP40/PBS as previously described [13]. The cells were then stained for Vpu or Env and

BST-2 using the antibodies described above. Images were obtained using a wide field fluorescence microscope fitted with a 100x objective (Olympus). For each field, a set of images was taken along the Z-axis, then processed with a "nearest neighbors" deconvolution algorithm (Slidebook; Imaging Innovations Inc). Single image planes are shown. Composite multi-channel images were assembled using Photoshop (Adobe Inc).

Luciferase-based reporter assay

96-well plates were coated with poly-D-lysine in water to enhance the retention of cells prior to plating. HEK293T Cells were transfected with the pGL4.32 vector containing five copies of an NF-kB response element (NF-kB-RE) that drive transcription of the luciferase reporter gene *luc2P*. Cells were transfected, incubated, and lysed within the same wells. 100 µl of Bright-Glo™ luciferase assay substrate (Promega) was added directly to the cells, which were mixed by pipetting. Following a 2-minute incubation period, the plates were read on a single-mode microplate reader (Molecular Devices) with an integration period of 0.5 seconds.

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III:

Results

Inhibition of clathrin-mediated endocytosis by AP180-C impairs the ability of HIV-1 Vpu and HIV-2 Env to antagonize cell surface BST-2.

The scission of endocytic vesicles from the membrane by dynamin 2 is crucial for the downregulation of BST-2 from the cell surface by either Vpu or HIV-2 Env [14]. To further implicate the endocytic pathway, and specifically, clathrin-mediated endocytosis, we sought to perturb the assembly of clathrin-coated vesicles using the C-terminal fragment of the clathrin assembly protein AP180. Over-expression of either full-length AP180 protein or just the C-terminal fragment of AP180 results in a block to clathrin-mediated endocytosis[15]. We transfected HeLa P4R5 cells (which constitutively produce endogenous BST-2) with AP180-C fragment in the form of a GFP-fusion protein (GFP-AP180-C). This allowed us to confirm the expression of the construct as well as determine the location of the protein within the cell. Expression of AP180C strongly inhibited the ability of Vpu and Env to decrease levels of surface-BST-2, as measured by flow cytometry (Fig. 1A and B). Curiously, expression of AP180-C decreased total cellular levels of both Vpu and Env, as measured by western blotting. Time-course experiments showed that this effect became more pronounced with time. To ensure that the inhibition of Vpu / Env activity by AP180-C was not due to a lack of expression, we collected the cells at various time points and found that at 8 hours post-transfection, expression of Vpu and Env was comparable in cells with or without AP180-C. Even only 8 hours after transfection, the inhibitory effect of Vpu and Env on surface BST-2 was clearly evident. Moreover, under these conditions, Vpu and Env activity was still abrogated by the expression of AP180-C. To further dispel any doubts about the level of

Vpu expression, we titrated the amount of Vpu plasmid transfected into cells. Even when the amount of Vpu plasmid was reduced to 0.05 µg (one-quarter of the typical 0.2 µg), the Vpu effect on BST-2 remained robust. Western blot analysis revealed that at 8 hours post-transfection, the expression of 0.2 µg of Vpu in the presence of AP180-C was comparable to the expression of 0.1 µg of Vpu without AP180-C. Despite similar cellular protein levels, Vpu activity was blocked by the presence of AP180-C. This shows that the inhibitory effects of AP180-C are not merely a consequence of its influence on Vpu expression.

Subcellular distribution of Vpu or Env are not affected by expression of AP180-C.

To determine whether AP180 influenced the subcellular distributions of Vpu and HIV-2 Env, we performed immunofluorescence microscopy. HeLa P4R5 cells were transfected with either Vpu or HIV-2 Env (together with HIV-1 Rev, a requisite for Env expression) and either GFP-AP180-C or GFP. Cells were stained for Vpu, HIV-2Env, and BST-2 24 hours post-transfection (Fig. 2). Vpu is typically found throughout the cytoplasm in endosomal structures, with a particularly high concentration near the cell center. Our images matched those obtained by others and suggest that Vpu is normally associated with the *trans*-Golgi network and recycling endosomes. [16,17]. BST-2 was partially co-localized with Vpu. HIV-2 Env was found distributed surrounding the nucleus in a feathery ring and in cytoplasmic puncta. These images suggest that HIV-2 Env is associated with the endoplasmic reticulum and with endosomes. AP180-C was found throughout the cytoplasm and often in a ring around the nucleus. AP180-C appeared to co-localize partially with Env. Importantly, AP180-C did not significantly

alter the distribution of Vpu or Env. AP180-C expression also did not affect the co-localization of BST-2 and Vpu. This data tell us AP180-C does not affect the targeting of Vpu or Env to their ultimate destinations within the cell. It is thus unlikely that AP180 serves directly as a cofactor for Vpu or Env.

AP180-C and dominant-negative dynamin 2 each decrease the endocytic rate of BST-2.

BST-2 is constitutively endocytosed and recycled to the cell surface at a rapid rate in HeLa cells [18,19]. We sought to measure the internalization rate of BST-2 from the cell surface and determine whether or not it was affected by our perturbations of clathrin-mediated-endocytosis. HeLa cells were transfected with plasmids expressing either dominant-negative Dynamin (dyn2K44A) or AP180-C. Following a 24-hour period, the cells were incubated with saturating amounts of labeled anti-BST-2 antibodies. The cells were then allowed to internalize the labeled BST-2. We fixed the cells at various time points and analyzed them using flow cytometry to measure the rate of disappearance of antibody-labeled BST-2 from the surface (Fig. 3). We found that the rate of internalization was significantly inhibited by both AP180-C and dyn2K44A. These results reinforce the role of clathrin-mediated-endocytosis in the trafficking of BST-2. The decreased rate of endocytosis could reduce the encounter rate between BST-2 and Vpu or Env. This would limit opportunities for Vpu or Env to direct BST-2 away from the surface or towards degratory pathways.

HIV-2 Env has no effect on endocytic rate of BST-2.

Several groups have documented the finding that Vpu does not affect the rate of endocytosis of BST-2 [18,19]. However, the effect of Env on the internalization of BST-2 had not been previously reported. To learn more about the mechanisms of Env activity, we measured the rate of BST-2 internalization +/- Env using flow cytometry. Cells were transfected with either HIV-1 Rev and HIV-2 Env ROD10 or with a matching amount of an empty-vector plasmid. Surface BST-2 was labeled with antibody and allowed to internalize under normal growth conditions. Env, like Vpu, had no effect on internalization rate (Fig. 4). This result ruled out the possibility that Env functioned by directing BST-2 endocytosis. Instead, it seems more likely that both Vpu and Env act at a post-endocytic step, perhaps by reducing the recycling of endocytosed BST-2 back towards the cell surface.

Disruption of the early endosomal sorting molecule Hrs has no effect on the inhibition of BST-2 by Vpu.

We sought to test our hypothesis of a Vpu-induced block to BST-2 recycling by perturbing several components of the recycling pathway. Hrs is an endocytic sorting molecule which is localized to early endosomes and helps direct movement of ubiquitinated proteins towards lysosomes[20]. Previous studies have shown that overexpression of Hrs inhibits the recycling of a ubiquitin-fused transferrin receptor and prevents early endosome fusion [21]. To antagonize Hrs activity, we obtained an anti-Hrs shRNA vector which encoded puromycin resistance. I first optimized the use of this system by empirically identifying the most effective shRNA sequence from four

candidates and confirming the time required for effective protein knockdown measured by western blot. The optimal plasmid was transfected into HeLa Z24 cells lacking puromycin resistance, allowing the use of selective media. The knockdowns proceeded for 72 hours, at which point cells were transfected with either Vpu or an empty-vector plasmid. Cells were collected the following day and analyzed by flow cytometry. Western blotting confirmed the effectiveness of the knock down (Fig. 5). The inhibitory activity of Vpu on BST-2 was not appreciably affected by the knock down of Hrs. This result contrasts with the findings of others, who reported that Hrs mediates Vpu-directed degradation of BST-2 [24]. The selection method employed was highly effective at preserving only cells expression the puromycin-resistance encoding plasmid and western blots showed the knockdown to be successful. However, even a small fraction of typical Hrs expression might be sufficient to maintain a near-normal phenotype. If our results are indeed correct, however, then it is unlikely that BST-2 downregulation is due to the activity of Hrs. Nevertheless, there are several other pathways by which proteins can be targeted to lysosomes for degradation.

Disruption of Rab-11 and Rab-22 GTPases has no effect on the inhibition of BST-2 by Vpu.

In addition to Hrs, we targeted Rab-11 and Rab-22, both of which are GTPases involved in directing protein trafficking and recycling. Rab-11 is implicated in directing recycling endosomes towards the cell surface [22]. Rab-22 has been shown to control the transport of cell surface proteins from sorting to recycling endosomes [23]. We obtained dominant-negative mutants of Rab-11 and Rab-22 which were either constitutively active

(Rab-11Q70L, Rab-22 Q64L) or inactive (Rab-11 S25N, Rab-22 S19N) and transfected cells with these constructs, along with wild-type Rab-11, Rab-22, Vpu, and GFP. Cells were analyzed by flow cytometry. Here, the expression of dominant-negative Rab GTPases did not affect the ability of Vpu to decrease levels of cell-surface BST-2 (Fig. 6). Therefore, it is unlike that Vpu acts at the final stages of returning BST-2 to the surface or at the intermediate stage of sorting endosomes. AS in the case if Hrs, our experimental manipulations might not have been sufficient to reveal an effect. However, even if they were, many other steps along the recycling pathway have yet to be explored as affected by Vpu.

BST-2-induced NF-κB activation is downregulated by Vpu and HIV-2 Env.

While the virion-restricting properties of BST-2 have been well documented, a different role has come to light. A recent study has identified BST-2 as an inducer of NF-κB activation [27]. This finding suggests that BST-2 serves as a cellular signaling molecule, though the mechanism and purpose have yet to be defined. We sought to study the effect of Vpu and HIV-2 Env on BST-2-mediated signaling by employing a luciferase-based assay. HEK 293T cells were transfected with a vector containing an NF-κB -driven luciferase reporter and either Vpu or Env. Cells were lysed in a buffer containing luciferase enzyme substrate and analyzed using a single-mode microplate reader. We found that NF-κB induction was proportional to BST-2 expression, and that both Vpu and Env strongly reduced the activation of NF-κB by BST-2 (Fig. 7A and B). To further examine this effect, we employed Vpu mutants which were defective for interaction with the β-TrCP E3 ubiquitin ligase complex (Vpu 2,6) or for interaction with

the transmembrane domain of BST-2 (Vpu AAA/FFF). While Vpu AAA/FFF maintained its inhibitory activity, Vpu 2/6 was defective. This finding suggests that the β-TrCP complex is required for the downregulation of NF-κB activity by Vpu while interaction with the transmembrane domain is not. In addition, mutations which removed BST-2 cytoplasmic ubiquitination sites increased the induction of NF-kB (Fig. 7C). Despite these changes, which inhibit Vpu's ability to cause degradation of BST-2, Vpu was still able to reduce NF-κB activation. These results lead us to propose that Vpu's ability to inhibit NF-κB activation is separate from its activity in causing BST-2 degradation. However, since Vpu can act as a saturation-inhibitor of β-TrCP, and β-TrCP is essential for NF-κB activation, careful dose response experiments are required to exclude a role for the interaction between Vpu and BST-2 and Vpu-induced degradation of BST-2 in the inhibition of NF-κB activation.

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IV:

Discussion

We have studied the role of the endocytic pathway in mediating the downregulation of BST-2 by HIV-1 Vpu and HIV-2 Env. Several previous studies had shown that the expression of BST-2 at the cell surface was decreased by the HIV accessory protein Vpu and the HIV-2 envelope glycotprotein [1]. We chose to investigate clathrin-mediated endocytosis because it is the most common pathway for the removal of proteins from membrane. In perturbing various steps of the endocytic pathway, we sought to determine where and how Vpu and HIV-2 Env might act on BST-2.

Inhibiting the normal function of AP180 by expressing AP180-C allowed us to specifically implicate clathrin-mediated endocytosis as a major factor in the activity of Vpu and Env. This information resonated with results obtained by collaborating lab members, who achieved a similar effect to AP180-C by expressingthe dominant-negative dynamin 2 mutant, dyn2K44A [14]. Vpu and Env activity was strongly inhibited by the expression of AP180-C while the localization of these viral proteins remained unaffected.

The simplest model for Vpu or Env activity would involve a direct interaction between BST-2 and Vpu or Env at the cell surface. However, Vpu was shown previously not to increase the rate of endocytosis of BST-2 [18,19]. We found that the expression of HIV-2 Env also did not enhance the rate of BST-2 endocytosis. Therefore, interactions between BST-2 and Vpu or Env likely takes place in an pre- or post-endocytic environment. By inhibiting endocytosis, we may have prevented BST-2 from reaching an l endosomal compartment where it could interact with and be acted upon by Vpu or Env. In support of this hypothesis, we found that both dyn2K44A and AP180-C inhibited the constitutive rate of endocytosis of BST-2. Furthermore, immunofluorescence

microscopy revealed that Vpu and Env are localized primarily in endosomes and other internal membranous compartments.

Several theories have been proposed regarding the post-endocytic fate of BST-2 in the presence of Vpu. Some groups have proposed that BST-2 is directed towards lysosomal degradation by Hrs [24]. However, previous studies by our group have shown that BST-2 co-localizes with transferrin, a maker of recycling endosomes, rather than with EGF, a marker of the ESCRT / lysosomal degratory pathway [14]. In our hands, shRNA mediated inhibition of Hrs did not affect the ability of Vpu to downregulate BST-2. Based on experimental evidence, we proposed that a block to recycling of endocytosed BST-2 was responsible for decreased surface levels of the protein.

To test this hypothesis, we perturbed two GTPases involved in the recycling pathway, Rab-11 and Rab-22. This also did not have a significant impact on Vpu activity. Nevertheless, there exists many different Rab family GTPases and other factors involved in the recycling pathway which have yet to be tested. Based on immunofluorescence microscopy, Vpu appears to be localized in endosomal compartments that include both recycling endosomes and the *trans*- Golgi network. Thus Vpu can plausibly affect the expression of newly-synthesized BST-2, in addition to recycling of endocytosed BST-2. Vpu and Env activity could take place at nearly any post-endosomal stage, and much exploration of this subject remains to be done.

In a separate area of study, we found that Vpu and Env antagonized the induction of NF-κB by BST-2. We have found that the expression of Vpu and Env modulate NF-κB activation, and that this effect is dependent upon the β-TrCP E3 ubuquitin ligase complex

in the case of Vpu. One experiment that could unite the themes of endosomal trafficking and signaling would be to measure BST-2 driven NF-κB induction in the presence of both Vpu and AP180-C. This might enable us to conclude the CME is required not only for Vpu's ability to reduce sufrace levels of BST2 but also for its ability to inhibit signaling by BST2. Our data also demonstrate that the Vpu effect on NF-κB induction is not dependent on its ability to interact with BST-2 or on presumed ubiquitination sites in BST-2, at least under the experimental conditions tested. At face value, this suggests that the inhibitory effect of Vpu on NF-κB induction is separate from the itsinhibitory effect on BST-2 expression. Similarly, Env is well known not to cause the degradation of BST-2, yet it is able to impede NF-κB activation [25,26].

These intriguing findings raises many questions about the role of BST-2 in combating enveloped viruses. Perhaps BST-2 serves as a signal to trigger an anti-viral response, since it is able to both interact with virus particle and induce NF-κB. It is also known, however, that NF-κB can be utilized to the virus's benefit, since HIV-1 contains sequences that recognize NF-κB in the enhancer region of its promotor [7]. Nevertheless, Vpu seems likely to modulate NF-κB activation in a manner beneficial to the virus.

In summary, we have shown that the counteraction of BST-2 by HIV-1 Vpu and HIV-2 Env is at least partially dependent upon clathrin-mediated endocytosis. Our experimental results suggest that Vpu and Env act on BST-2 in a post-endocytic step, possibly through a block to recycling. Additionally, Vpu and Env antagonize NF-κB induction through a potentially separate mechanism. These findings provide new areas of

exploration, as the exact purpose and mechanisms of their activities have yet to be determined.

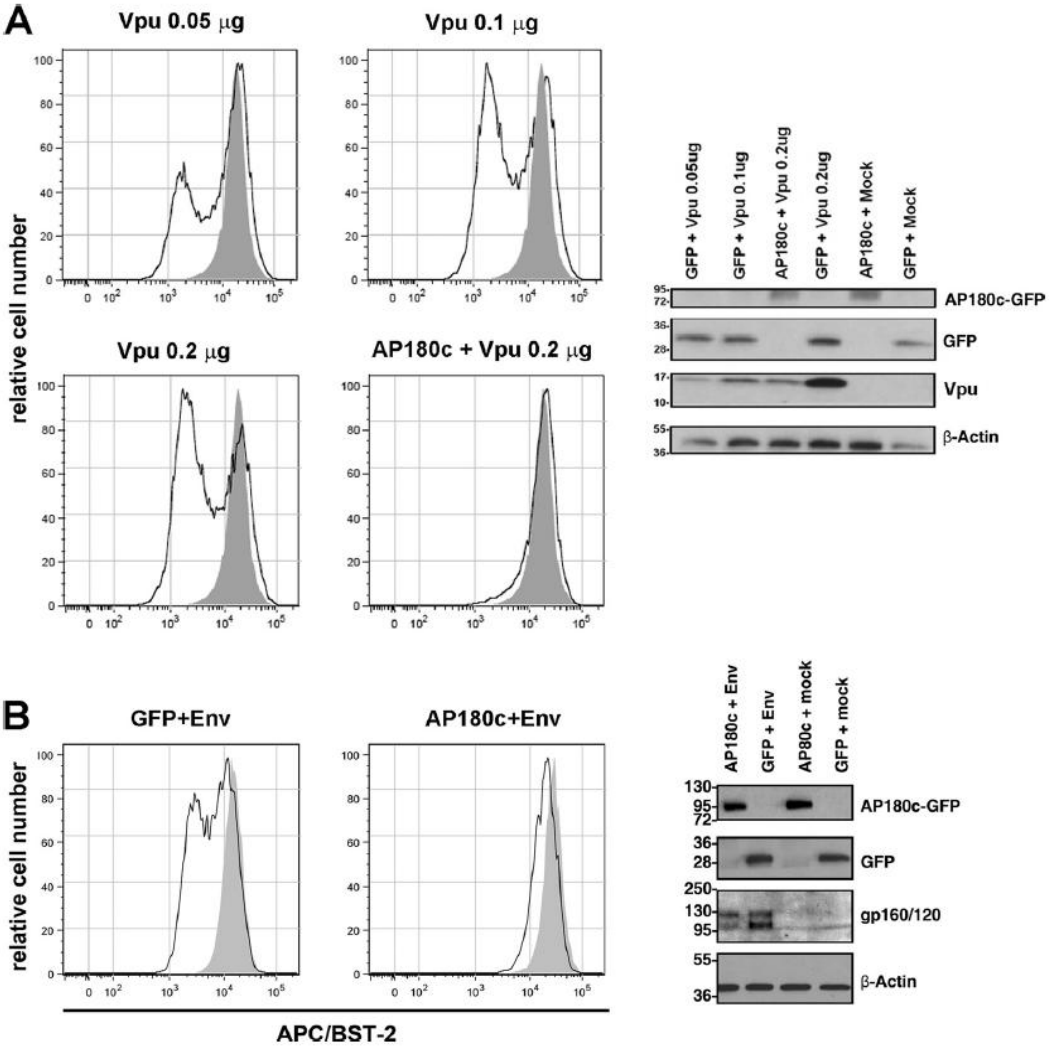

Fig. 1. The C-terminal fragment of the clathrin assembly cofactor AP180 inhibits the downregulation of cell surface BST-2 by Vpu and HIV-2 Env. (A) Inhibition of Vpu-mediated downregulation of cell surface BST-2 by the C-terminal fragment of the clathrin assembly cofactor AP180 (GFP–AP180-C). Cells (HeLa) were transfected to express the AP180 C-terminal fragment fused to GFP (AP180-C; 0.7 μg of plasmid) with Vpu (0.2 μg of plasmid) or GFP alone (0.06 μg of plasmid) with Vpu (0.05, 0.1, or 0.2 μg of plasmid). Total plasmid DNA was made (up to 1.6 μg using pcDM8). Eight hours later, the cells were stained for surface BST-2 and analyzed by two-color flow cytometry. GFP-positive cells are shown as open histograms in the plots of the relative number of cells versus fluorescence intensity of APC/BST-2. Shaded histograms are derived from cells expressing neither Vpu nor GFP–AP180-C. The cells were also analyzed for the expression on GFP–AP180-C, GFP, Vpu, and actin by immunoblotting. (B) Inhibition of Env-mediated downregulation of cell surface BST-2 by AP180-C. Cells were transfected as described above, except that instead of Vpu, HIV-2 Env (0.4 μg of plasmid) was expressed along with HIV-1 Rev (0.4 μg of plasmid). Shaded histograms represent cells not expressing Env but expressing GFP (left) or GFP–AP180 C (right); open histograms represent cells expressing Env and GFP (left) or Env and GFP–AP180-C (right). The cells were also analyzed for the expression on AP180-C, GFP, Env (gp160/120), and actin by immunoblotting.

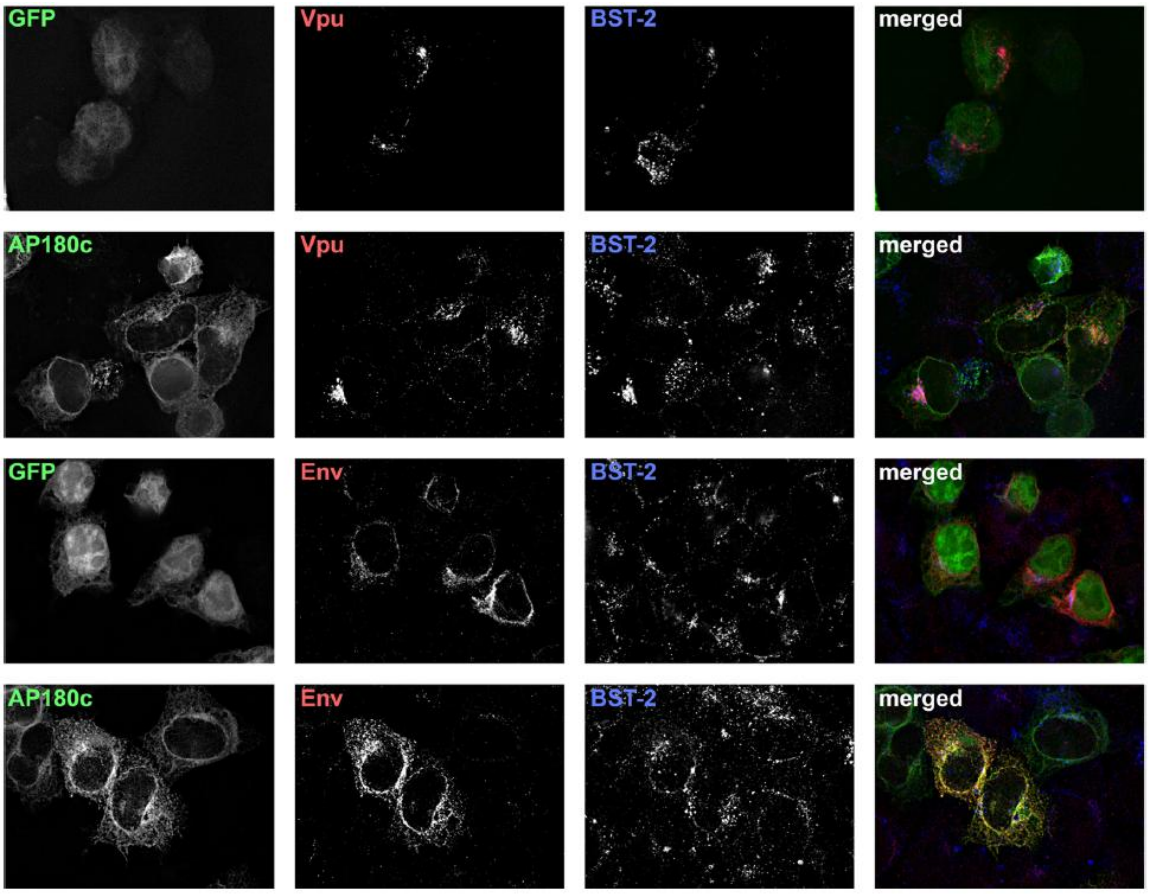

Fig. 2. The C-terminal fragment of the clathrin assembly cofactor AP180 does not affect the subcellular distribution of Vpu or Env. Cells (HeLa) were transfected to express either the AP180 C terminus fused to GFP (AP180-C; 0.35 µg of plasmid) or GFP (0.03 µg of plasmid), along with either Vpu (0.1 g of plasmid) or HIV-2 Env with HIV-1 Rev (0.1 µg of each plasmid); total plasmid was made up to 0.8 µg in each case with the empty vector pCDM8. The next day, the cells were fixed, permeabilized, and stained for Vpu or Env, together with BST-2, and imaged using wide-field fluorescence microscopy. A Z series of images was obtained, and these were processed by a deconvolution algorithm before export of the single-plane images shown. In the "merged" images, GFP proteins are shown in green, Vpu or Env is red, and BST-2 is blue. Overlap between the viral proteins and BST-2 appears purple, whereas overall between the viral proteins and AP180-C or GFP appears yellow.

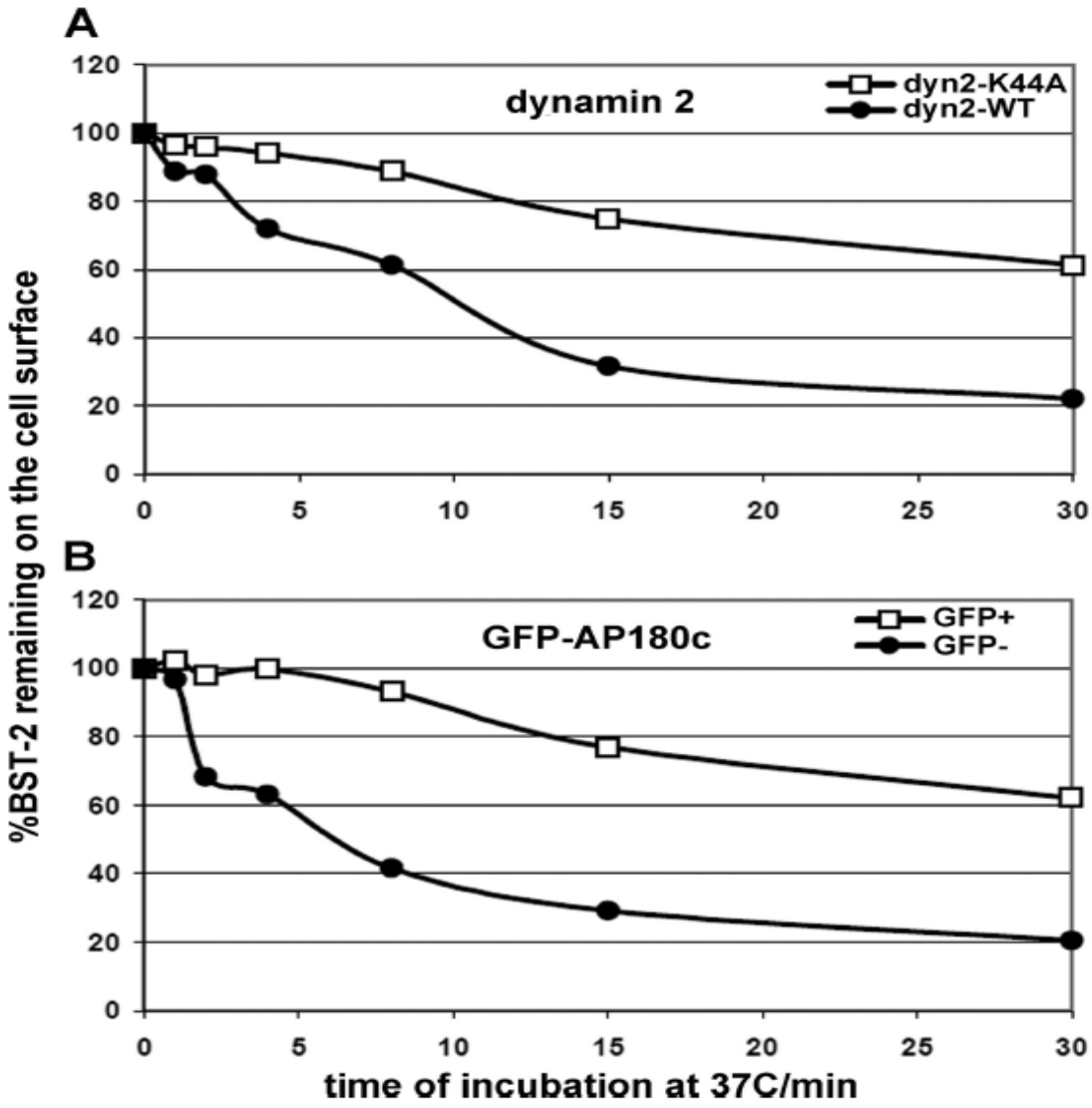

Fig. 3. Dominant negative dynamin 2 or AP180-C inhibits the endocytic rate of BST-2. (A) Dynamin2 K44A. Cells (HeLa) were transfected to express either GFP-dyn2K44A (0.9 µg of plasmid) or GFP-dyn2 wild type (WT). The next day, a flow cytometric assay was used to measure the rate of internalization of BST-2. After labeling of the cell surface at 4°C with antibody to BST-2, the cells were incubated for various times at 37°C before staining with a fluorophore-conjugated secondary antibody and analysis by two-color flow cytometry. The fraction of BST-2 remaining on the cell surface versus the time of incubation at 37°C is graphed, using the mean BST-2/APC fluorescence intensity of the GFP-positive cells and normalizing to the amount of BST-2 present at time zero in each case. (B) AP180-C. Cells (HeLa) were transfected to express GFP–AP180-C (0.7 µg of plasmid). The next day, the flow cytometric assay described above was used to measure the rate of internalization of BST-2. The GFP - curve is derived from the GFP-negative cell population (not expressing AP180- C), whereas the GFP + curve is derived from the GFP-positive cell population (expressing AP180-C).

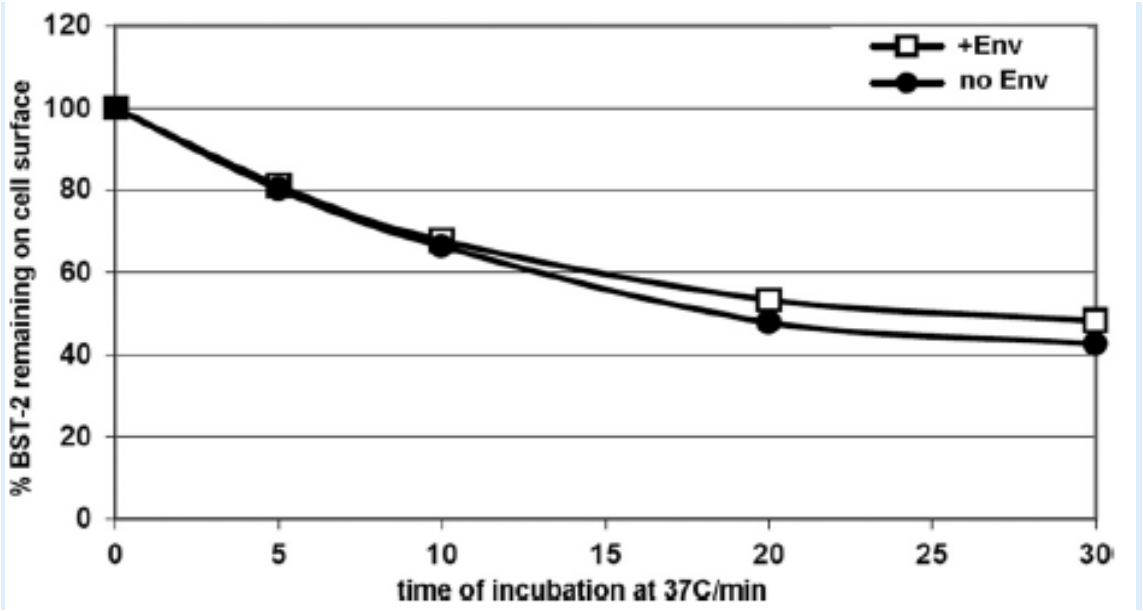

Fig. 4. Effect of HIV-2 Env on the endocytic rate of BST-2. A flow cytometric assay was used to measure the effect of HIV-2 Env on the rate of internalization of BST-2. The assay was performed as described previously for Vpu (19), except that the cells (HeLa) were transfected to express GFP (0.06 _g of plasmid) either with or without HIV-2 Env and HIV-1 Rev [0.8 µg of each plasmid or 1.6 µg of pcDM8 control (no Env)]. After labeling of the cell surface at 4°C with antibody to BST-2, the cells were incubated for various times at 37°C before staining with a fluorophore-conjugated secondary antibody and analysis by two-color flow cytometry. The fraction of BST-2 remaining on the cell surface versus the time of incubation at 37°C is graphed in the presence and absence of HIV-2 Env, using the mean BST-2/APC fluorescence intensity of the GFP-positive cells and normalizing to the amount of BST-2 present at time zero in each case.

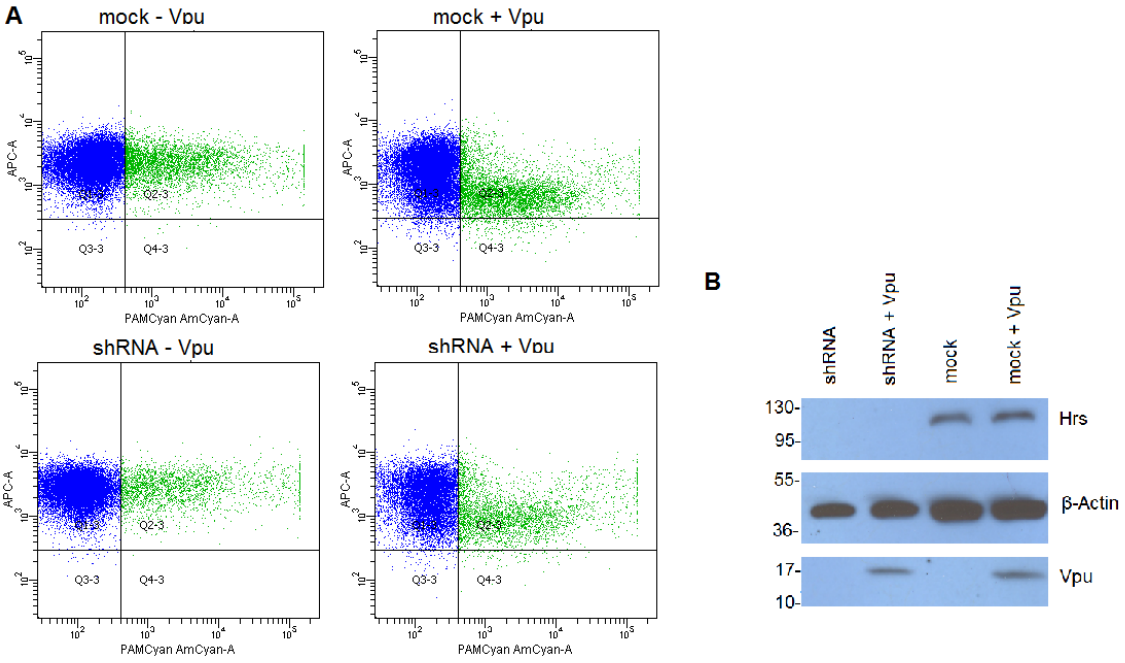

Fig. 5. Disruption of the early endosomal sorting molecule Hrs has no effect on the inhibition of BST-2 by Vpu. (A) 3-color flow cytometry was used to measure surface BST-2 levels in cells that were transfected with either an anti-Hrs shRNA vector (HGS 002) or an empty vector (TR3008, mock). Both vectors contained GFP. HeLa Z24 cells were initially transfected with the HGS 002 or mock and allowed to grow for 72 hours. A secondary transfection in which a marker (pmCyan) and either Vpu or an empty vector (pCDNA 3.1) then took place. 24 hours later, the cells were collected, stained for surface BST-2 and analyzed by 3-color flow cytometry. (B) Cells were analyzed by immunoblotting to ensure effectiveness of the knockdown.

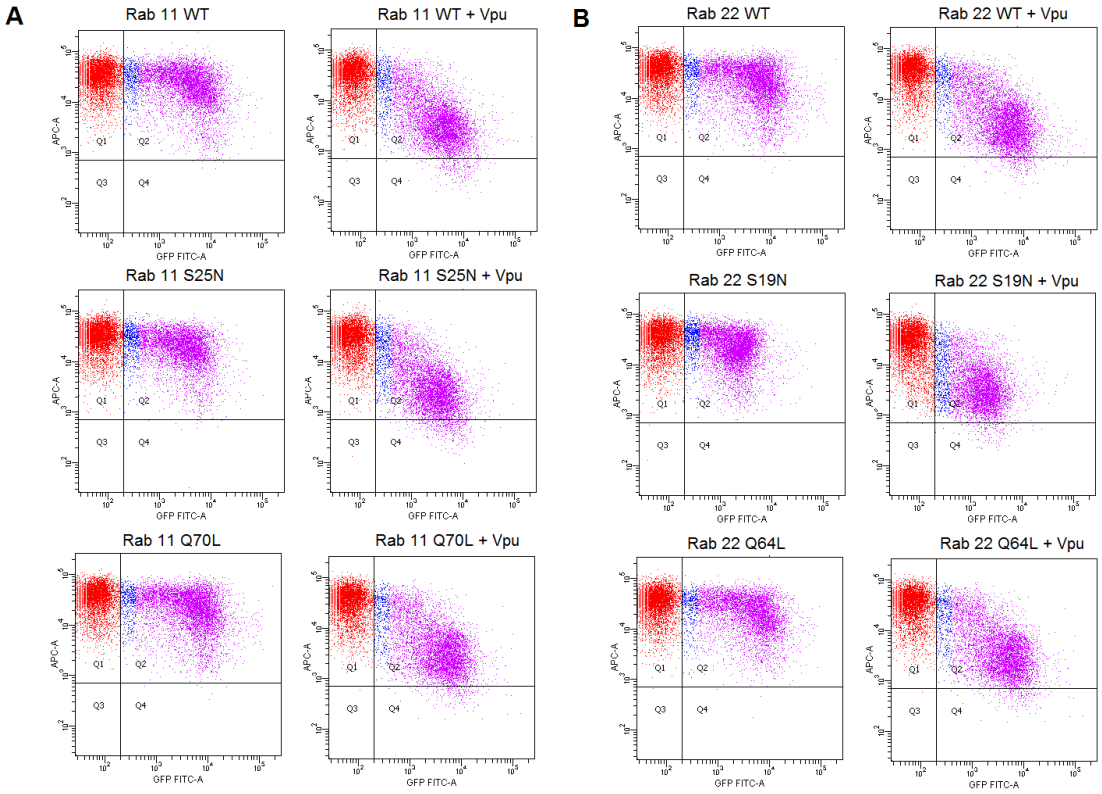

Fig. 6. Disruption of Rab-11 and Rab-22 GTPases has no effect on the inhibition of BST-2 by Vpu. (A) HeLa P4R5 cells were transfected with Rab-11 WT, S25N (dominant-negative), or Q70L (constitutively active). Vpu was co-transfected with each Rab condition. Cells were collected 24 hours post-transfection, stained for surface BST-2 and analyzed by two-color flow cytometry.(B) HeLa P4R5 cells were transfected with Rab-22 WT, S19N (dominant-negative), or Q64L (constitutively active). Vpu was co-transfected with each Rab condition. Cells were collected 24 hours post-transfection, stained for surface BST-2 and analyzed by two-color flow cytometry

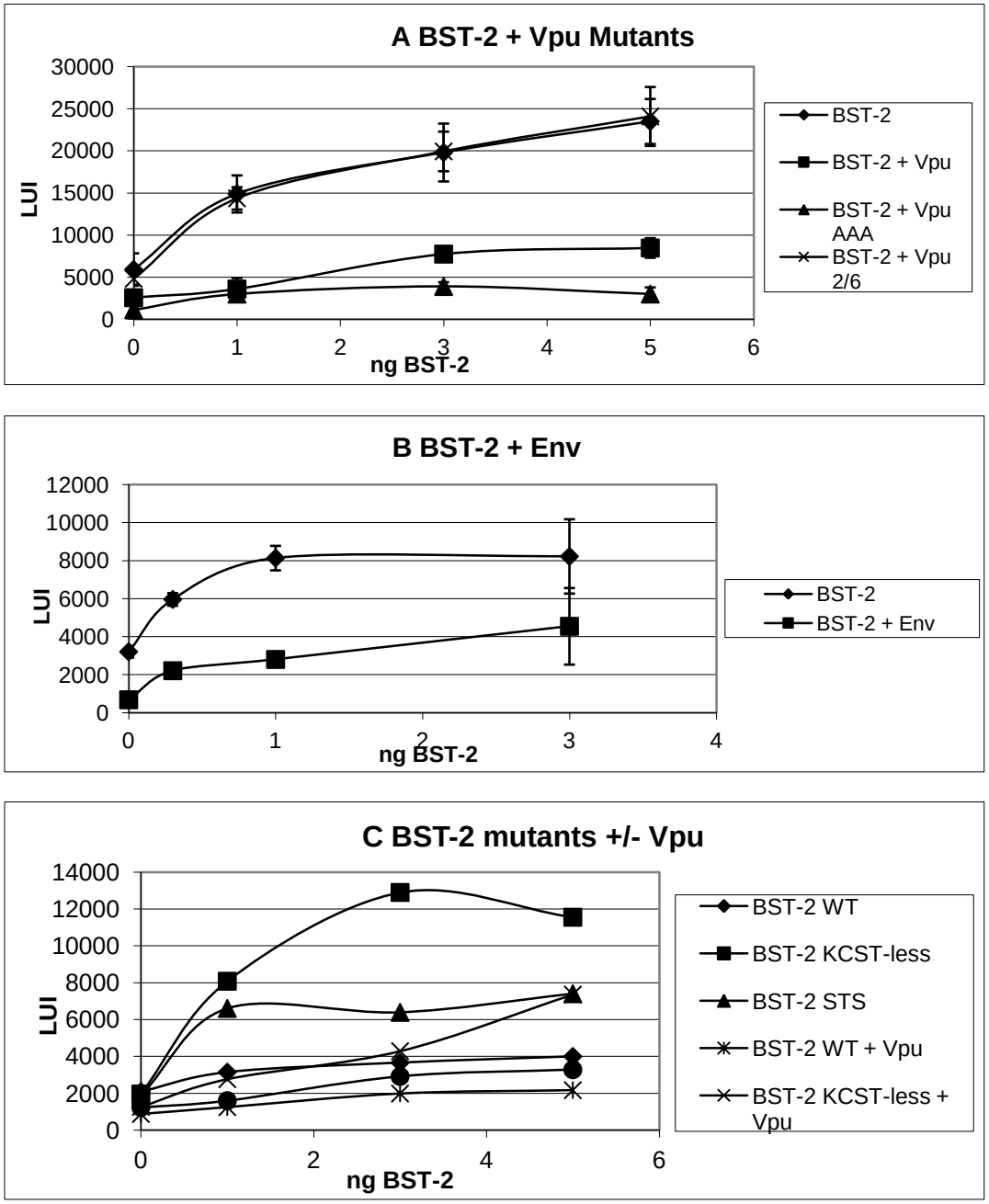

Fig. 7. BST-2-induced NF-κB activation is downregulated by Vpu and HIV-2 Env. In all experiments, cells were transfected with a NF-κB-responsive luciferase reporter and lysed in a buffer containing luciferase substrate. All cells were analyzed on a single-mode microplate reader. (A) Vpu downregulates NF-κB induction by BST-2 in an β-TrCP dependent manner. Hela 293T cells were transfected with BST-2 and wild-type Vpu, Vpu 2/6 (deficient for β-TrCP interaction) , or Vpu AAA (deficient for interaction with BST-2 TMD). (B) HIV-2 Env downregulates NF-κB induction by BST-2. Hela 293T cells were transfected with BST-2 and wild-type HIV-2 Env from the ROD10 isolate. (C) Removal of BST-2 ubiquitination sites causes upregulation of NF-κB activation but still allows inhibition by Vpu. Cells were transfected with wild-type BST-2, STS, or KCST-less mutants lacking proposed ubiquitination sites.

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