Antiviral Properties of BST-2 and Kaposi's Sarcoma-associated Herpesvirus Countermeasures

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CERTIFICATE OF APPROVAL

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Dedicated in the memory of Cindy L. Hines.

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Upon infection a virus elicits a great number of responses from the host cell, and in order for a successful infection to occur, the virus must employ various mechanisms to evade these immune responses. One such immune evasion method of Kaposi's sarcoma-associated herpesvirus (KSHV) is the viral E3 ubiquitin ligase, K5. K5 is known to downregulate the major histocompatability complex (MHC) class one, which is part of the immune response. Another possible target for downregulation by K5, BST-2, had been suggested. BST-2 has been shown to inhibit viral egress of some enveloped viruses by tethering nascent virions to the surface of the cell. Here the relationship of K5 and BST-2, and that of BST-2 and KSHV infection, is examined. It is determined that K5 has the ability to downregulate BST-2, alone or in the context of KSHV infection. The lysines on the cytoplasmic N-terminal tail of BST-2 are ubiquitinated by K5 after BST-2 leaves the ER, after which, BST-2 is then destined for the lysosome for degradation. In addition to this, it is demonstrated that BST-2 has the ability to restrict KSHV viral release in the

absence of K5. By showing that BST-2 is a bona fide substrate of K5, the importance of K5 in overcoming the innate immune response is further elucidated.

Chapter 1
Introduction

1.1 Background

1.1.1 *Herpesviridae*

Herpesviridae is a family of viruses that has a fairly gigantic membership, including viruses capable of infecting a wide variety of hosts, including oysters, penguins, salmon, and of course, humans. Herpesviruses all share common properties; they are large enveloped viruses, with a linear double-stranded DNA genome averaging in the range of one to two hundred kilobases, the capsid is icosahedral and surrounded by a matrix of proteins called the tegument. Also common is the ability to maintain latent infection within the host cell, during which no progeny virus is produced, but a few viral proteins and the viral genome are present. There are eight known herpesviruses that are found in humans. These include herpes simplex 1 and 2 (HSV-1 and HSV-2) which cause oral and genital herpes, human cytomegalovirus (HCMV), varicella-zoster virus (VZV), the causative agent of chicken pox, Epstein-Barr virus (EBV), human herpesviruses 6 and 7 (HHV-6 and HHV-7), and HHV-8, also known as Kaposi's Sarcoma-associated herpesvirus (Fig. 1.1.1) [1].

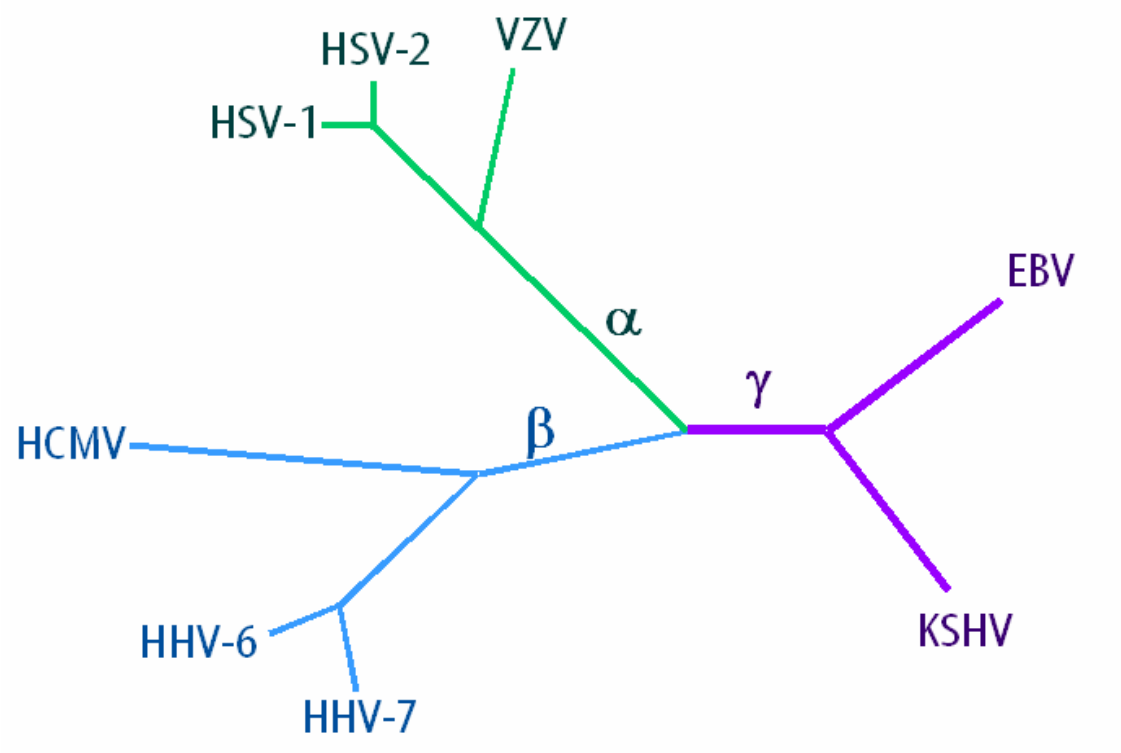

Figure 1.1.1

Human herpesvirus phylogenetic tree

Alpha herpesviruses include herpes simplex virus 1&2 and varicella-zozter virus. Beta herpesviruses include human herpesvirus 6&7 and human cytomegalovirus. Kaposi's sarcoma associated herpesvirus and Eptein-Barr virus are gamma herpesviruses.

1.1.2 Kaposi's Sarcoma-associated Herpesvirus

Kaposi's sarcoma (KS) is a multiple pigmented sarcoma of the skin that appears as purple-colored dermal or mucosal lesions, though tumors can also be present in lungs, liver, and intestines. These lesions are made of a network of endothelial spindle-shaped cells that form irregular microvascular channels. Kaposi's sarcoma was not known to be associated with viral infection until 1994 when Chang et al. identified DNA in the sarcoma that they found to be related to herpesviruses [3]. In most populations KSHV is quite uncommon. Seroprevalence is measured by the amount of antibodies for KSHV that are detectable in the blood of a sampled population. Throughout North and South America, Europe, and Asia the seroprevalence is between two and five percent. However, even of the infected, only one of ten thousand actually develop symptoms of KS [4, 5]. In the Mediterranean the seroprevalence is higher, about ten percent, and is highest in Sub-Saharan Africa at around forty percent, where it is the most commonly reported neoplasm [5, 6]. KS is much more common in the immunocompromised, such as the elderly, organ transplant recipients, and especially in those with AIDS. At one point in 1981, 48% of gay men with AIDS had developed KS, and as such it became recognized as a sentinel disease of AIDS [7].

In addition to KS, KSHV can cause two other diseases, both of which are quite rare. Both affect B cells, multicentric Castleman disease (MCD) is a B cell lymphoproliferative disorder, and primary effusion lymphoma (PEL), a non-Hodgkin's B cell lymphoma [8, 9].

KSHV is a gamma herpesvirus, like Epstein-Barr virus, and more specifically a gamma-2-herpesvirus (rhadinovirus) like the simian virus, herpesvirus saimiri. The genome of KSHV is linear and the coding region is about 140kb in length, flanked by 801bp terminal repeats [10]. The genome encodes approximately 90 open reading

frames. KSHV encodes 75 ORFS (labeled as ORFs1-75) that share a great deal of homology with herpesvirus saimiri, most of which encode for proteins that are important in generating viral progeny and establishing latency. In addition to these 75 genes, KSHV encodes 15 genes that are unique to KSHV, called K1-15 [10]. Many of these genes share homology with cellular genes, suggesting that KSHV has appropriated them up from host cells at some point [11]. Of these genes that share homology, many of the corresponding proteins play a part in either immune evasion, tumorigenesis or both [12].

1.1.3 Latent and Lytic Infection

A remarkable aspect of herpesviruses is the ability after primary infection to maintain persistent latent infection. KSHV is no exception; even in tumors only about three percent of cells undergo lytic replication [13, 14]. During latency very few viral proteins are expressed, including latency associated nuclear antigen 1 (LANA-1), viral cyclin (v-Cyc), and viral Fas-associated death domain (FAAD) like interleukin-1β-converting enzyme (FLICE) inhibitory protein (v-FLIP). Producing so few viral genes during latent infection is one of the ways that KSHV prevents detection by the host immune system [15]. All three are encoded on a 5.32kb transcript, splicing to a 1.7kb transcript encodes v-FLIP and v-Cyc [16]. Other proteins are found in some, but not all, latently infected cells. One of these is kaposin, which is found at low levels in PEL cells and in some spindle cells [17, 18]. In addition, KSHV encodes four viral interferon regulatory factors (vIRFs), and two of them, vIRF-1 and vIRF-3, are occasionally detected in latently infected tissues [19]. The two vIRFs that are present in some latent cells and the three universal latent genes all play important roles in maintaining latency and promoting immune evasion during latent infection.

During latency, LANA-1 maintains many aspects of the infection. LANA-1 is encoded by ORF73, a 222-234 kDa protein [20, 21]. The genome of the virus exists in the cell's nucleus as a circularized episome during latency [22]. LANA-1 physically

associates the episome with the host cell's DNA [23]. The association with cellular chromatin results in the episome being replicated during cell replication by the cell's own machinery and allows the viral genome to be maintained in cells without undergoing lytic replication. The major latent promoter is activated by LANA-1 [24]. LANA-1 is extremely important for maintaining latency and has a great deal of transcriptional control.

One of the viral proteins that LANA-1 keeps transcriptional tabs on is the replication and transcription activator (RTA) [25]. RTA is an immediate early gene encoded by ORF50 that serves to reactivate lytic infection during latency. [26]. At about 110 kDa, RTA has an N-terminal DNA binding and dimerization domain, a C-terminal activation domain and two nuclear localization signals [26, 27]. RTA is extremely important to the state of infection of KSHV, as it regulates a great deal of the viral genes that are expressed, including itself [28]. Upon initial infection, RTA upregulates expression of LANA, thus causing latency [29]. However, when reactivation occurs, RTA is responsible for transcriptional activation of other lytic genes to drive the lytic cycle to completion to ultimately produce virions [26, 27].

Cells express a wide variety of proteins that keep strict control over the cell cycle, interfering with these leads to uncontrolled proliferation. Several of the methods that KSHV employs while maintaining latency can lead to tumorigenesis [12]. LANA-1 can cause a reduction in p53-mediated apoptosis [30]. LANA-1 has also been shown to bind to the chromatin remodeling protein RING3, which can lead cells to enter S phase [31]. LANA-1 and v-Cyc also regulate tumor suppressor retinoblastoma protein (Rb), which also causes cells to enter S phase [30, 32, 33]. v-FLIP activates NF-κB to help with the survival of infected cells by inhibiting apoptosis [34, 35]. In addition, vIRF-1 induces a growth arrest by inhibiting transforming growth factor-β (TGF-β) [36]. Kaposin activates part of the p38 pathway, which upregulates inflammatory responses and cytokine production [37]. KSHV also expresses twelve micro RNAs; including miRK-1,

which represses the expression of a protein with known tumor suppressor functions, p21 [38]. These are just several of the mechanisms by which KSHV prolongs the life of the infected cell.

1.1.4 Immune Evasion Mechanisms

KSHV has developed an impressive array of methods to evade the immune system of the host cell it infects. About a quarter of the KSHV genome encodes proteins that play a role in down regulating some aspect of the immune system [39].

A large part of the manner in which KSHV works against cellular immunity is by interfering with interferon (IFN). The IFN response is an extremely important part of innate immunity, and without countermeasures the IFN system can control an infection almost entirely without help from the adaptive immune response [40]. IFNs are secreted cytokines that have antiviral effects. Type one IFNs, IFN-α and IFN-β, are induced upon viral infection. Induction of IFN-β occurs when nuclear factor κB (NF-κB) and IRF3 in the cytoplasm are phosphorylated, which ultimately reveals nuclear localization signals, and are translocated to the nucleus [41]. Upon binding to the IFN-β promoter, p300 also associates and recruits transcriptional machinery. IFN produced during viral infection leads to the induction of several IFN regulatory factors (IRF-1, IRF-7, IRF-9). IRF-7 enhances the transcription of the primary IFN genes and allows transcription of the secondary IFN genes, IRF7 can also bind to the IFN-β promoter and enhance transcription [42, 43]. The IFN signaling cascade is ultimately responsible for inhibiting viral replication through the expression of Interferon stimulated genes (ISGs).

KSHV encodes four proteins that have homology to IRF, vIRFs1-4, which play a part in regulating the IFN response by acting on different aspects of IFN induction. These viral IRFs (vIRFs) work on several levels to alter the expression of IFN and IFN signaling. All four vIRFs are expressed during lytic infection, and vIRF-1 and vIRF-3

are also found in some latently infected cells [19]. IFNα–, IFNβ–, and IFNγ– induced responses are blocked by the expression of vIRF-1 [44]. Both vIRF-2 and vIRF-3 decrease activation of NF-κB [45, 46]. While cellular IRFs interact directly with DNA, vIRFs associate with transcriptional activator p300. vIRF-1 regulates IRF3 mediated transcription by associating directly with p300 [47]. vIRF-2 also binds to p300 and works to inhibit IFN-inducible gene expression that would be controlled by IRF-1, IRF-3 and IFN-stimulated gene factor 3 [45]. Additional KSHV proteins also interefere with the IFN pathway. RTA and ORF45 inhibit IRF7. RTA inhibits expression of type I IFN by ubiquitinating IRF7 so that it undergoes proteasomal degradation [48]. ORF45 inhibits type I IFN during early stages of the infection by preventing phosphorylation of IRF7 and thus inhibiting its translocation to the nucleus where it would mediate type I IFN transcription [49]. All these mechanisms together are quite effective in controlling IFN signaling.

Modulating the host T-cell response during infection is extremely important as T-cells play a vital role in immune response. T helper (T_H) cells display the glycoprotein CD4, and cytotoxic T cells (T_C) have CD8 on the surface. T_H cells interact with the major histocompatibility complex class II (MHC) molecules, which present antigens that have been acquired from outside of the cell by endocytosis. Depending on the type of cytokines produced, two different immune responses may occur. T_H1 response results in the activation of macrophages, whereas the T_H2 response plays a part in the activation of B cells. T_C cells recognize MHC class I, which present antigens that were generated within the cell [50].

KSHV has evolved a variety of strategies to modulate the T-cell response. One such strategy employs viral interleukin-6 (vIL-6). vIL-6 is one of the most abundant viral mRNAs expressed in lytic PEL cells [14]. RTA not only upregulates vIL-6, but also cellular IL-6 [51]. Like its human counterpart, vIL-6 promotes hematopoiesis [52]. The T_H-1 cell response has also been shown to be inhibited by vIL-6, but vIL-6 upregulates

T_H-2 cell responses, which are less effective [53]. vIL-6 also induces vascular endothelial growth factor (VEGF), thereby causing angiogenesis and effusion formation [52, 54]. KSHV has three viral chemokine homologs, called viral CC-chemokine ligands 1-3 that affect the chemokine-mediated immune response [55, 56]. vCCL1 and vCCL3 antagonize host CC-chemokine receptor 8 (CCR8) and CCR4, which attracts T_H2-cells [57, 58]. However, vCCL2 has the ability to inhibit signaling of a wide variety of chemokine receptors of T_H1-cells. KSHV, like several other herpesviruses, also encodes a homolog to CD200, vCD200. CD200 is a leukocyte glycoprotein that binds to the receptor CD200R, which is present on myeloid and T-cells [59]. vCD200 binds to CD200R, and by so doing inhibits myeloid cell activation and reduces the production of T_H1-cell associated cytokines [59, 60]. Together all these mechanisms are thought to be important for regulating the cellular immune response.

Another important way KSHV likely remains undetected by Tc cells is by downregulating the antigen presenter MHC class I. Since MHC class I presents viral antigens that are produced within infected cells, many viruses have evolved counter-mechanisms. In KSHV-infected cells, MHC class I is downregulated at the transcriptional level by vIRF1 and vFLIP [61]. KSHV employs two ubiquitin E3 ligases, K3 and K5, that target MHC class I for degradation. Because of their presumed role in immune evasion, these ubiquitin ligases are also known as modulators of immune recognition (MIR) [62].

1.1.5 K5

K3 and K5, like most other viral ubiquitin ligases, are of the RING (really interesting new gene) ligase family (Fig. 1.1.2 A) [63]. K5 and K3 are specifically RING-CH, defined by a C4H3 Cys-His conformation [64]. An interesting aspect of viral RING-CH ligases is that they all downregulate MHC class I [63]. These ligases are present in a variety of herpes and poxviruses, and bear a strong resemblance to the

cellular MARCH (membrane associated RING-CH) proteins, suggesting these proteins may have been co-opted by the viruses from cells [65].

K5 is a type IV transmembrane protein with its N-terminal RING domain facing the cytoplasm followed by two transmembrane domains [66]. RTA activates transcription of K5 during lytic infection [26]. K5 is able to ubiqutinate its substrates for either lysosomal or proteasomal degradation [62, 67]. Substrates of K5 are generally ubiquitinated in post-ER compartments, which are then sorted to lysosomes via the multivesicular body pathway [68, 69].

1.1.6 Targets for Downregulation by K5

K5 has the ability to downregulate a fair number of targets. K5 thus inhibits the innate immune responses of infected cells and, by removing the signals that the cell is infected, adaptive immune responses to infected cells. This is achived by reducing T cell responses and avoiding antiviral cytokine responses. As mentioned above, viral ubiquitin ligases downregulate MHC class I; K5 does this by increasing endocytosis of MHC class I and marking it for degradation [70]. Interestingly it has been reported that K5 and K3 not only ubiquitinate lysine residues but, in their absence, also other amino acid groups such as serine, cysteine and threonine in the class 1 molecule. K5 targets preferentially lysines or cysteines that are closer to the transmembrane in cytoplasmic tail and K3 targets the aminoacids that are at least 15 amino acids away from the transmembrane [71]. In addition to MHC class I, K5 ubiquitinates several other substrates that play a number of roles within the cell. Downregulation of CD1d results in reduced activation of CD1d-restricted T-cell activation [72]. Another target for downregulation by K5 is PECAM (platelet endothelial cell adhesion molecule), which reduces PECAM dependent endothelial cell migration [67]. In addition, ICAM-1 (inter-cellular adhesion molecule 1) and B7-2 which are important for promoting T-cell priming, are also targeted for

downregulation by K5 [73]. Downregulation of adhesion molecules may play a role in KSHV-induced tumorigenesis.

A screen to identify more substrates of K5 through a quantitative global membrane proteomics approach identified bone marrow stromal antigen 2 (BST-2), activated leukocyte cell adhesion molecule (ALCAM) and Syntaxin-4 as additional targets [74]. At the time the function of BST-2 was unknown, but now as it has been shown to play an important role in innate immunity, it is a particularly interesting target for downregulation by K5.

1.1.7 BST-2

BST-2 was identified by two laboratories independently in 1994. Both found it to be related to B cells. Goto et al. determined it to be a marker of terminally differentiated B cells whereas Ishikawa et al. described that it might play a roll in pre-B cell growth [75, 76]. It has not been until much more recently that the role of BST-2 was further elucidated.

The *BST-2* gene is 543bp and encodes 180 amino acids. BST-2 has a transmembrane domain close to the N-terminus [77]. Since BST-2 transmembrane protein the fact that it has 19 hydrophobic residues on the N-terminus serves as a signal sequence that would be recognized by the signal-recognition particle (SRP) for the ribosome to be associated with the translocon in the ER [77, 78]. As a type II transmembrane protein, BST-2 has a signal-anchor sequence that causes the N-terminus to remain associated with the cytoplamic side of the membrane and the C-terminus to pass through [79]. BST-2 undergoes several post-translational modifications. The C-terminus of BST-2 is very hydrophic, with about 17 hydrophibic residues, in which is a signal sequence to aquire a glycosyl-phosphatidylinositol (GPI) anchor [77]. The GPI anchor is added in the ER and signals the trafficking of BST-2 to the cell surface, where

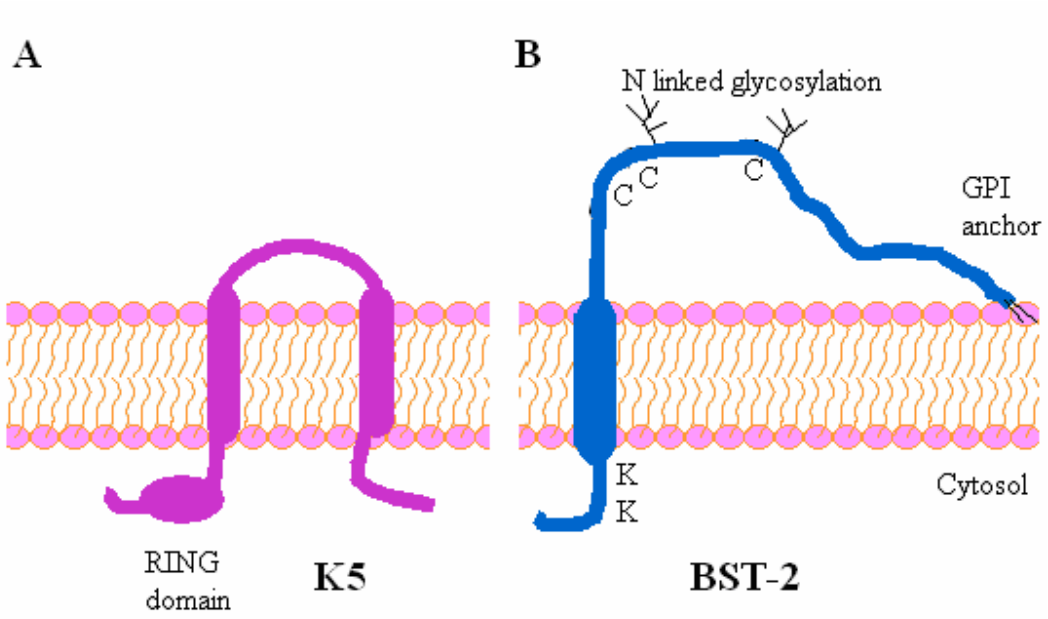

Figure 1.1.2

Structure of K5 and BST-2

(A) The ubiquitin ligase activity of K5 comes from the N terminal RING domain, K5 has two transmembrane domains. (B) BST-2 has a short N terminus with two lysines. There are two sites of N linked glycosylation, and three cysteins that can form disulfide bonds. The C-terminus associates with lipid rafts in the membrane through a GPI anchor.

the GPI anchor will associate with cholesterol rich lipid rafts on the extracellular side of the plasma membrane [78, 79]. In the ER BST-2 is glycosylated at two sites, at residues 65 and 92, first oligosaccharides are transferred to the asparagines in BST-2 in the ER, these then undergo further trimming and additions in the Golgi [77-79]. BST-2 has three cysteines within the extracellular coiled coil which form dimers through disulfide bonds (Fig. 1.1.2 B)[80]. BST-2 is not only located in the cell membrane, but in other intracellular compartments as well, such as the trans-Golgi network and recycling endosomes [77]. BST-2 traffics via clathrin-mediated endocytosis [81]. The uncommon properties of the structure of BST-2 make it a very interesting protein to study.

1.1.8 BST-2 as a Viral Tether

In 2006 BST-2 was identified as being present on the surface of cells that had been treated with interferon-α [82]. In addition, it was found that the promoter for *BST-2* has tandem repeats of the interferon response element [83]. Both of these findings indicate that BST-2 plays a role in the innate immune response. This was quite interesting as it had been shown by our group that the viral ubiquitin ligase K5 was capable of downregulating BST-2 [74]. In recent years a plethora of new information appeared regarding BST-2, starting with work done in HIV-1. It had been shown that the HIV-1 accessory protein Vpu was essential for viral egress in certain cell types, however the mechanism of its action was not quite understood [84]. The mechanism was clarified when Neil et al. sought to identify how a virus lacking Vpu formed virions that remained at the cell surface and were eventually taken up by endosomes [85]. It was shown that viral particles gathered on the surface of cells lacking Vpu through electron microscopy [85]. It was determined that there was a membrane associated protein that tethered the virions to the surface of the cell; therefore they termed this protein “tetherin.” Microarray analysis identified BST-2 as one of the proteins involved [85]. It has been suggested that tethering to the cellular membrane is accomplished by BST-2 being

present in the virion and forming dimers with a BST-2 in the cell membrane through disulfide bonds [80, 86].

The possibility that BST-2 could work on a number of enveloped viruses seems entirely feasible, considering that it has been shown that Vpu enhances release of other retroviruses [87]. As such, in the time since BST-2 tethering properties have first been suggested, more viruses have been examined to further elucidate the role that BST-2 plays in inhibiting viral egress.

1.1.9 The Relationship of BST-2 and Enveloped Viruses

One important area of research is determining if the way BST-2 tethers HIV-1 to the surface of the cell is exclusive to HIV-1 or if this mechanism is common across viral species. Jouvenet et al. examined a variety of unrelated viruses to determine if this was indeed the case, they found that not only did BST-2 have an effect on viral egress with another lentivirus, SIV, but also other retroviruses (alpharetrovirus, betaretrovirus and gammaretrovirus) [88]. The gammaretroviruses, porcine endogenous retrovirus (PERV) and murine leukemia virus (MLV), have also been found to have a decreased amount of viral release from cells expressing BST-2 [89]. Endogenous sheep betaretroviruses (enJSRVs) have also been shown to have restricted viral egress in the presence of BST-2 [90]. In addition to other retroviruses, it has been found that BST-2 had an effect on release of virus like particles (VLPs) in filoviruses, Marburg and Ebola, and in the arenavirus Lassa. [88, 91]. The fact that so many unrelated viruses are affected by BST-2 suggests that this mechanism of retaining viruses to the cell surface might apply to any enveloped virus.

1.1.10 Methods of Downregulation of BST-2

Since not all viruses have equivalents to Vpu, the question arises as to how other viruses downregulate BST-2. Amongst retroviruses alone a variety of mechanisms have

been determined. HIV-2 appears to sequester BST-2 in the trans-Golgi network in a manner that involves the envelope protein (Env) [92]. In SIV the negative regulatory factor (Nef) appears to be responsible for antagonizing BST-2 [93]. Filovirus mechanisms to overcome BST-2 have also been examined. By testing four proteins known to be important for viral egress on cells expressing BST-2, the glycoprotein was identified as restoring egress, though exactly how has yet to be further clarified [94]. The mechanisms of other have yet to be elucidated.

1.2 Experimental Goals

Because BST-2 has been shown to have an effect on many enveloped viruses and K5 is known to downregulate BST-2, we seek to further clarify the relationship between K5 and BST-2 as a potential immune evasion mechanism of KSHV. One of the manners by which we intend to do so is by examining the amount of KSHV released from cells that express BST-2 and viral release when K5 is or is not present, compared against cells that do not express BST-2. We also seek to confirm that BST-2 is being ubiquitinated and undergoing degradation. We intend to verify that BST-2 is indeed able to inhibit viral egress, and that BST-2 is a bona fide target of down regulation by K5.

Chapter 2

Molecular Mechanism of BST2/Tetherin Downregulation by K5/MIR2 of Kaposi's Sarcoma-Associated Herpesvirus

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2.1 Abstract

K3/MIR1 and K5/MIR2 of Kaposi's sarcoma-associated herpesvirus (KSHV) are viral members of the membrane-associated RING-CH (MARCH) ubiquitin ligase family and contribute to viral immune evasion by directing the conjugation of ubiquitin to immunostimulatory transmembrane proteins. In a quantitative proteomic screen for novel host cell proteins downregulated by viral immunomodulators, we previously observed that K5, as well as the human immunodeficiency virus type 1 (HIV-1) immunomodulator VPU, reduced steady-state levels of bone marrow stromal cell antigen 2 (BST2; also called CD317 or tetherin), suggesting that BST2 might be a novel substrate of K5 and VPU. Recent work revealed that in the absence of VPU, HIV-1 virions are tethered to the plasma membrane in BST2-expressing HeLa cells. By targeting BST2, K5 might thus similarly overcome an innate antiviral host defense mechanism. Here we establish that despite its type II transmembrane topology and carboxy-terminal glycosylphosphatidylinositol (GPI) anchor, BST2 represents a bona fide target of K5 that

is downregulated during primary infection by and reactivation of KSHV. Upon exit of the protein from the endoplasmic reticulum, lysines in the short amino-terminal domain of BST2 are ubiquitinated by K5, resulting in rapid degradation of BST2. Ubiquitination of BST2 is required for degradation, since BST2 lacking cytosolic lysines was K5 resistant and ubiquitin depletion by proteasome inhibitors restored BST2 surface expression. Thus, BST2 represents the first type II transmembrane protein targeted by K5 and the first example of a protein that is both ubiquitinated and GPI linked. We further demonstrate that KSHV release is decreased in the absence of K5 in a BST2-dependent manner, suggesting that K5 contributes to the evasion of intracellular antiviral defense programs.

2.2 Introduction

Bone marrow stromal cell antigen 2 (BST2) was recently identified as a host cell restriction factor that prevents the release of retroviral and filoviral particles from infected host cells [88]. Human immunodeficiency virus type 1 (HIV-1) counteracts this antiviral function of BST2 by expressing the viral auxiliary protein VPU [85, 95]. In the absence of VPU, virus particles are prevented from budding off the cellular membrane in cells that express BST2, resulting in virions being tethered to the plasma membrane. BST2 was therefore renamed tetherin [85], although questions still remain as to whether BST2 acts as the actual tether and whether BST2-dependent tethering occurs in all BST2-expressing cell types [96]. Independently, BST2 was shown to be induced by type I and type II interferons (IFNs) [82], suggesting that BST2 is part of the innate antiviral response triggered in infected cells.

Using a quantitative membrane proteomic approach, we observed that BST2 is underrepresented in plasma membranes from cells expressing not only VPU [97] but also the K5 protein of Kaposi's sarcoma-associated herpesvirus (KSHV) [74]. K5 is a viral homologue of a family of cellular transmembrane ubiquitin ligases, termed membrane-associated RING-CH (MARCH) proteins [65], that mediate the ubiquitination of the cytoplasmic portion of transmembrane proteins (reviewed in reference 40). Each member of this family targets a subset of cellular membrane proteins with both unique and shared specificities [74, 98]. One of the functions of cellular MARCH proteins is to modulate antigen presentation by mediating the ubiquitin-dependent turnover of major histocompatibility complex (MHC) class II molecules in dendritic cells, B cells, and monocytes/macrophages [66, 99]. In contrast, viral homologues of MARCH proteins encoded by KSHV, murine herpesvirus 68, and the leporipoxvirus myxomavirus all share the ability to mediate the destruction of MHC-I (reviewed in reference 16) but not MHC-II molecules. Thus, one of the functions of the viral proteins is to promote viral escape from immune clearance by CD8+ T lymphocytes [100]. Furthermore, each viral MARCH homologue specifically eliminates additional host cell proteins, so each plays multiple

roles in viral pathogenesis. KSHV carries two viral MARCH proteins, K3 and K5, also known as MIR1 and MIR2, which both support viral escape from T-cell, NK-cell, and NKT-cell recognition by eliminating the corresponding ligands from the surfaces of infected cells (reviewed in reference 38). In endothelial cells (ECs), K5 additionally downregulates EC-specific adhesion molecules that play an essential role in the formation of adhesive platforms and adherens junctions [67, 101]. Since Kaposi's sarcoma is a tumor of EC origin, K5 might thus also contribute to tumorigenesis by disrupting normal EC barrier function and by modulating the interaction of ECs with inflammatory leukocytes.

The downregulation of BST2 by K5 further suggests that K5 also counteracts innate antiviral responses, which might benefit KSHV. However, most transmembrane proteins targeted by viral or cellular MARCH proteins are type I transmembrane proteins that belong to the immunoglobulin superfamily. In contrast, BST2 is a type II transmembrane protein that is also glycosylphosphatidylinositol (GPI) anchored [77]. Thus, BST2 has a short cytoplasmic amino terminus followed by an outside-in transmembrane domain, a large glycosylated extracellular portion, and a GPI anchor. The additional propensity of BST2 to form homodimers [83] was speculated to be crucial for the tethering function of BST2 in that self-association of BST2 molecules in the viral envelope with plasma membrane BST2 could prevent viral exit [87]. The unusual topology of BST2 and its multimerization raised the question of whether BST2 is a bona fide target of K5 or whether its downregulation is a downstream effect of K5 eliminating other transmembrane proteins. Additionally, it is not clear whether BST2 would be downregulated in the context of a normal viral infection and, particularly, whether virally expressed K5 would be able to overcome the high expression levels of BST2 observed upon IFN induction. We now demonstrate that KSHV efficiently downregulates IFN-induced BST2 both during primary infection and upon reactivation from latency in ECs. IFN-induced BST2 is ubiquitinated by K5 upon exiting the endoplasmic reticulum (ER) and is rapidly degraded by a pathway that is sensitive to proteasome inhibitors but

resistant to inhibitors of lysosomal acidification. These data suggest that despite its unusual topology, BST2 is directly targeted by K5. We further demonstrate that BST2 reduces KSHV release upon inhibition of K5 expression by small interfering RNA (siRNA), suggesting that BST2 is part of the IFN-induced innate immune response to KSHV. Thus, in addition to contributing to viral evasion of cellular immune responses and remodeling EC function, K5 also counteracts the innate immune defense of the host cell.

2.3 Materials and Methods

2.3.1 Viruses and cell culture

Dermal microvascular endothelial cells (DMVECs) were immortalized by transfection with human papillomavirus (HPV) E6 and E7 and infected with KSHV as described previously [102]. KSHV was obtained from reactivated BCBL-1 cells as described previously (37). Replication-deficient adenovirus expressing N-terminally (K3) or C-terminally (K5) FLAG-tagged inserts under the control of a Tet transactivator-dependent promoter and Ad-RTA were described previously [101]. Ad-TET was obtained from David Johnson, Oregon Health and Science University.

HeLa cells were infected with rKSHV.219 (kindly provided by J. Vieira). rKSHV.219 expresses green fluorescent protein (GFP) under the control of a constitutive promoter and red fluorescent protein under the control of a lytic promoter and exhibits puromycin resistance [103]. Latently infected cells were plated in six-well plates and were treated with control, K5, or BST-2 siRNA, using Lipofectamine 2000 transfection reagent following the manufacturer's protocol. The transfection was repeated after 8 h. Ad-RTA was used to reactivate the virus in latently infected cells. Forty-eight hours after infection, supernatants were collected, filtered, and used to infect 293 cells. Cells were analyzed by flow cytometry after 48 h to measure GFP fluorescence.

To generate stable cell lines expressing BST2, the human bst-2 cDNA was amplified as an NheI/BamHI fragment by a PCR using the Pfu enzyme (Stratagene, San Diego, CA) and inserted into the lentiviral vector pCDH-CMV-MCS-EF1-Puro (System Biosciences, Mountain View, CA). BST2 and its mutants with a hemagglutinin (HA) tag at position 146 (BST2-HA) and lysine-to-arginine replacements at positions 18 and 21 (BST2-KR) were generated by primer-directed mutagenesis using PCR. Lentiviral supernatants were produced via triple transfection of 293T cells with the pHP-dl-N/A

packaging construct, the pHEF-VSVG envelope construct (both constructs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Lung-Ji Chang [9]), and one of the lentiviral clones described above. Transfections were performed using Effectene (Qiagen, Germantown, MD), with a plasmid ratio of 6:1:3 (packaging construct:envelope construct:lentiviral clone). After 48 h, the supernatants were collected and lentiviruses were purified through a 0.8-µm filter. Stable cell lines expressing BST2, BST2-HA, BST2-HA-KR, and BST2-KR were generated by lentiviral transduction and puromycin selection (0.3 µg/ml). An empty pCDH vector was used as a control.

2.3.2 Antibodies and reagents

Mouse anti-human BST2 (HM 1.24) was kindly provided by Chugai Pharmaceutical Co., Ltd. (Kanagawa, Japan), and was previously characterized [83]. This antibody was used for cytofluorometry (1:200), immunofluorescence (1:100), immunoprecipitation (1 µg/ml of lysate), and immunoblotting (1:2,000). Anti-ubiquitin (clone P4D1) and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human HLA antibody W6/32 was obtained from Sigma (St. Louis, MO), and anti-HA antibody was obtained from Covance Research Products Inc. (Denvar, PA). Anti-K5 antibody was described previously [104].

The following reagents were used at the indicated concentrations. Concanamycin A (ConA) (working concentration, 50 nM) was purchased from Sigma (St. Louis, MO). MG132 was obtained from Boston Biochem (Cambridge, MA) and used at a final concentration of 20 µM. Human β-IFN (PBL Biomedical Laboratories, Piscataway, NJ) was used at 500 U/ml, and recombinant human tumor necrosis factor alpha (TNF-; R&D Systems, Minneapolis, MN) was used at 10 ng/ml. Dominant-negative VPS4 (GFP-E228Q) and wild-type GFP-VPS4 plasmids were obtained from Wes Sundquist [105].

2.3.3 Cell surface protein biotinylation

Cell surface proteins were biotinylated with EZ-Link NHS-SS-biotin following the manufacturer's protocol (Pierce, Rockford, IL). Briefly, cells were washed three times with ice-cold phosphate-buffered saline (PBS), and primary amines of the membrane proteins exposed to the exterior of the cells were biotinylated with NHS-SS-biotin for 30 min at 4°C. The cells were washed and lysed immediately with a nonionic detergent. Labeled proteins were isolated with immobilized NeutrAvidin (Pierce, Rockford, IL). The bound proteins were released by incubating the resin with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 50 mM dithiothreitol.

2.3.4 Metabolic labeling and immunoprecipitation

Cells were starved in Dulbecco's modified Eagle's medium deficient in methionine and cysteine for 30 min and then pulse labeled for 15 min with 80 µCi/ml L-[35S]methionine and 24 µCi/ml L-[35S]cysteine (Express protein labeling mix; PerkinElmer, Boston, MA). The label was chased for various times with full medium containing an excess of normal cysteine and methionine. Cells were lysed in SDS buffer (1% NP-40), and antigens were immunoprecipitated with 1 µg/ml of antibody. Precipitates were either left untreated or treated with 1 µg of endo-β-N-acetylglucosaminidase H (endo H) or 500 U of N-glycosidase F (PNGase F; New England Biolabs, Ipswich, MA). The proteins were separated in a 10% SDS gel, dried, and exposed to Kodak Biomax MR film. Treatment with MG132 (20 µM) or ConA (50 nM) was done for 12 h after transduction with Ad-K5 for 4 h.

2.3.5 Immunoblotting of immunoprecipitated BST2

To solubilize BST2, 6 x 105 cells were lysed in 250 µl of 0.6% SDS in PBS at room temperature for 20 min and then scraped off and diluted, using 750 µl of 1.2% Triton X-100. The cell lysate was cleared of debris by centrifugation at 10,000 x g for 10 min at 4°C. BST2 was immunoprecipitated using 1 µg of HM1.24 antibody. The immunoprecipitate was washed in 0.2% Triton X-100, resuspended in gel loading buffer (10% glycerol, 5% β-mercaptoethanol, 2% SDS, 50 µM Tris, pH 6.8, and 0.02% bromophenol blue), and separated by 10% SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (Waters Ltd., Milford, MA) and probed with primary antibodies for 1 h at room temperature, followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Antibody Solutions). Membranes were washed in 0.1% Tween 20 in PBS. The proteins were detected using a SuperSignal West Femto chemiluminescence kit (Thermo Scientific, Rockford, IL).

2.3.6 qPCR

Total mRNA from cells was isolated and purified using RNeasy (Qiagen). Specific primers for BST2, K5, and β-actin were designed using Primer 3 software (BST2 primers were CCGTCCTGCTCGGCTTT [forward] and CCGCTCAGAACTGATGAGATCA [reverse], K5 primers were ACAAGGACCGTCAATTCGATG [forward] and TGCCATACCGACGGCC [reverse], and β-actin primers were TCACCCACACTGTGCCCATCTACGA [forward] and CAGCGGAACCGCTCATTGCCAATGG [reverse]). Transcript levels were determined by quantitative real-time reverse transcriptase PCR (qPCR), using SYBR green dye incorporation (AB Applied Biosystems, Warrington, United Kingdom) and AmpliTaq Gold DNA polymerase in an ABI Prism 7900HT sequence detection system (AB Applied Biosystems, Warrington, United Kingdom). The comparative threshold cycle method was

used to derive the change in BST2 expression between different treatments, using β-actin as an internal standard [2].

2.4 Results

2.4.1 KSHV downregulates IFN-induced BST2 during primary infection

Using quantitative proteomics, we previously observed that BST2 was underrepresented in membrane preparations of K5-expressing HeLa cells [74]. The mass spectrometric result was independently verified using a commercially available anti-BST2 antiserum that was of limited specificity and could be used only in immunoblots. To independently verify that BST2 is downregulated by K5 in a cell type that can be infected with KSHV, we studied BST2 expression in HPV E6/E7-immortalized DMVECs (E-DMVECs) [102], using the BST2-specific monoclonal antibody HM1.24 (kindly provided by Chugai Pharmaceuticals) [83]. In untreated DMVECs, we observed low levels of BST2 expression at the cell surface (Fig. 2.4.1A). However, a significant increase in the BST2 surface level occurred upon treatment with IFN-β, consistent with previous reports that BST2 is inducible by IFN [82]. A similar induction was observed upon treatment with TNF- (Fig. 2.4.1A). In contrast, prior transduction of DMVECs with an adenovirus expressing K5 (Ad-K5) reduced IFN-β-induced BST2 expression, whereas transduction with Ad-K3 or Ad-TET alone had no effect on IFN induction of BST2 (Fig. 2.4.1B). These data confirm our previous observations with HeLa cells, which express BST2 constitutively, and demonstrate that K5 is able to downregulate BST2 expression upon IFN induction in cells representative of Kaposi's sarcoma.

Upon de novo infection of E-DMVECs, KSHV establishes latent infection (37). Prior to establishment of latency, however, K5 is transiently expressed [106], although protein levels in the majority of cells are typically below the limits of detection by standard methods [107]. Upon primary infection of DMVECs with KSHV, a significant reduction of BST2 surface expression was observed in IFN-induced DMVECs (Fig. 2.4.1B). The percentage of cells expressing low levels of BST2 corresponded to cells that were LANA-1 positive (not shown). To determine whether BST2 downregulation was the

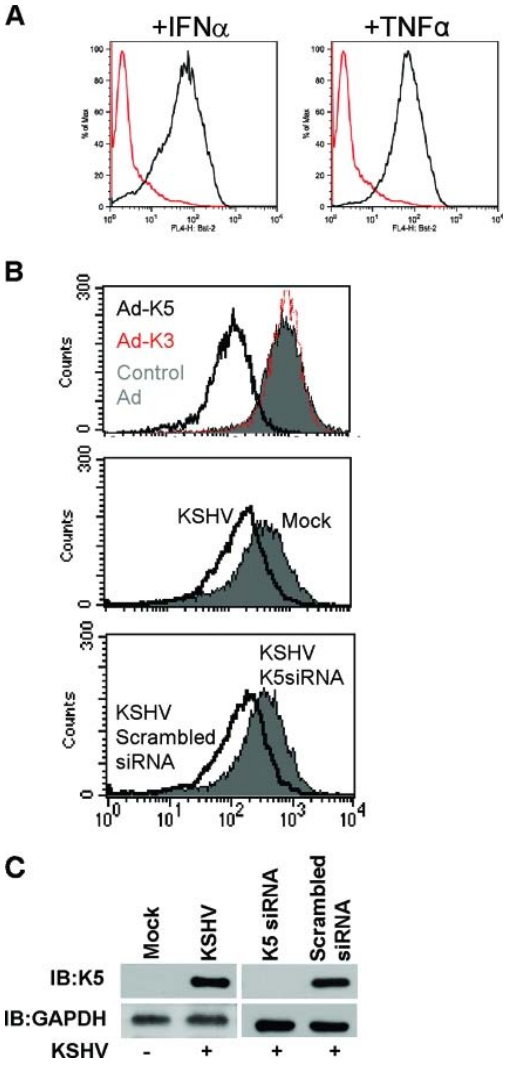

FIG. 2.4.1

K5 downregulates IFN-induced BST2 in DMVECs infected with KSHV.

(A) BST2 is induced by IFN-β and TNF-α in DMVECs. E-DMVECs were treated with 500 U/ml of human IFN-β or 10 ng/ml TNF-α for 24 h or were left untreated (red) prior to flow cytometry with anti-BST2 (HM1.24). (B) K5 downregulates IFN-β-induced BST2 during de novo infection with KSHV. (Top) E-DMVECs were transduced with Ad-K5 (black), Ad-K3 (red), or Ad-TET (gray) for 24 h prior to treatment with IFN-β and flow cytometry for BST2. (Middle) E-DMVECs were infected with KSHV (white) or mock infected (gray) prior to treatment with IFN-β and staining for BST2. (Bottom) E-DMVECs were treated with K5-specific (white) or scrambled (gray) siRNA and infected with KSHV prior to treatment with IFN-β and staining for BST2. (C) K5-specific siRNA inhibits K5 protein expression during primary infection. Duplicate samples from the middle and lower parts of panel B were used to confirm K5 knockdown by K5 siRNA. Cells were lysed and immunoblotted with anti-K5 or anti-GAPDH antibody.

consequence of transient K5 expression in primary infected DMVECs, we pretreated DMVECs with siRNA to K5 or control siRNA, as described previously [101, 107], prior to KSHV infection. As shown in Fig. 2.4.1B, BST2 was downregulated in control siRNA-treated cells, but KSHV had no effect on BST2 levels upon treatment with K5 siRNA. Reduction of K5 protein expression by siRNA was verified by immunoblotting (Fig. 2.4.1C). Taken together, these data suggest that during de novo infection, KSHV downregulates BST2 by expressing K5.

2.4.2 KSHV inhibits IFN-induced BST2 expression upon reactivation from latency

To examine whether KSHV is able to prevent IFN-induced BST2 upregulation during latency or upon reactivation from latency, we studied BST2 expression in latently infected or reactivated E-DMVECs. Latently infected E-DMVECs were generated as described previously (37). As shown in Fig. 2.4.2A, latently infected cells did not counteract IFN-induced BST2 upregulation, consistent with a lack of K5 expression during established latency. Latent virus can be reactivated experimentally by introduction of the viral transactivator RTA, which induces lytic genes, including the K5 gene [26]. Upon reactivation of KSHV by transduction of latently infected cells with Ad-RTA, we observed a significant inhibition of IFN-induced BST2 surface expression, whereas Ad-TET was unable to prevent BST2 induction (Fig. 2.4.2A). This correlated with the strong induction of K5 expression (Fig. 2.4.2B). Since KSHV carries several modulators of IFN signaling [108], it was possible that reactivated KSHV prevented BST2 induction at the transcriptional level. However, qPCR of BST2-specific transcripts revealed a strong induction of a BST2-specific message upon IFN induction in both latently infected and reactivated samples (Fig. 2.4.2B). Therefore, we concluded that during reactivation, BST2 expression is inhibited at a posttranscriptional level, consistent with K5-mediated degradation. In contrast to the case with primary infected cells, however, we did not observe increased BST2 expression upon treatment of reactivated cells with K5-specific

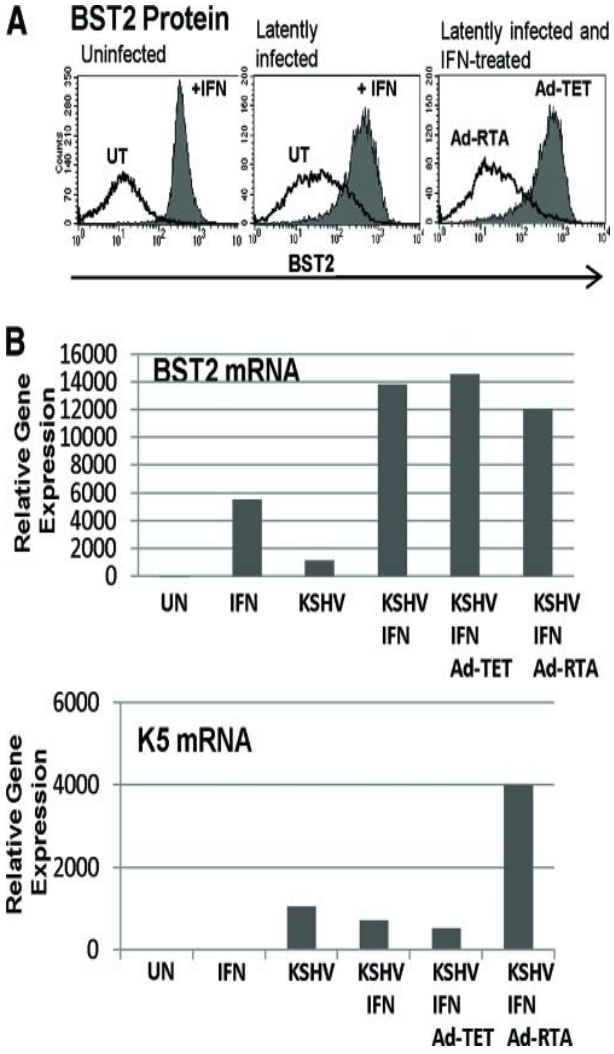

Figure 2.4.2

KSHV inhibits induction of BST2 protein, but not mRNA, upon reactivation.

(A) BST2 downregulation upon viral reactivation. (Left) Uninfected E-DMVECs were treated with IFN-β for 24 h (+IFN; gray) or were left untreated (UT; black) prior to being stained with anti-BST2 (HM1.24). (Middle) E-DMVECs latently infected with KSHV were treated with IFN-β (+IFN; gray) or left untreated (UT; black). (Right) Latently infected E-DMVECs were transduced with Ad-RTA (black) or Ad-TET (gray) prior to treatment with IFN-β for 24 h. (B) KSHV does not inhibit induction of BST2 mRNA by IFN-β. BST2 (top) and K5 (bottom) mRNA levels were quantified by qPCR for cells treated as described for panel A. Results were normalized to a β-actin control, and changes compared to untreated, uninfected E-DMVECs were calculated using the comparative threshold cycle method as described previously [2].

siRNA due to incomplete gene knockdown (data not shown). Since very low levels of K5 are sufficient to downregulate target proteins [107], presumably due to the catalytic nature of ubiquitin ligases, the efficient reactivation of K5 expression by Ad-RTA seems to outcompete the K5-specific siRNA. However, the fact that BST2 expression is inhibited at a posttranscriptional level strongly suggests that K5 is responsible for this effect upon viral reactivation from latency.

2.4.3 BST2 is degraded upon exiting the ER

BST2 is an unusual transmembrane protein that has a type II transmembrane topology while also carrying a carboxy-terminal GPI anchor. As such, it is very different from all other transmembrane proteins that are targeted by K5, i.e., type I transmembrane proteins belonging to the immunoglobulin superfamily. To determine how BST2 is downregulated by KSHV K5, we studied the fate of BST2 in the presence of K5. The effects of K5 on cell surface-expressed and total BST2 were monitored by cell surface biotinylation and immunoblotting of total cell lysates, respectively. In IFN-induced DMVECs transduced with Ad-TET, cell surface biotinylated BST2 appeared as several protein bands that corresponded in molecular size to monomeric (36 kDa), dimeric (>64 kDa), and multimeric forms of BST2, as described previously [83] (Fig. 2.4.3A). In the presence of K5, all forms of BST2 were undetectable, suggesting that K5 efficiently prevented surface expression of IFN-induced BST2. A similarly strong reduction was observed for total levels of BST2 by immunoblotting of total SDS lysates (Fig. 2.4.3B), suggesting that IFN-induced BST2 was efficiently eliminated by K5.

To facilitate the study of the K5-mediated degradation of BST2 by use of a commercially available antibody, we generated human fibroblasts stably expressing wild-type BST2 or modified BST2 containing an internal HA epitope tag at a nonconserved site [85]. BST2-HA was initially synthesized as an endo H-sensitive protein of approximately 24 kDa that was converted into an endo H-resistant form of approximately 36 kDa (Fig. 2.4.3C). Upon PNGase F treatment, the 36-kDa protein was converted to the

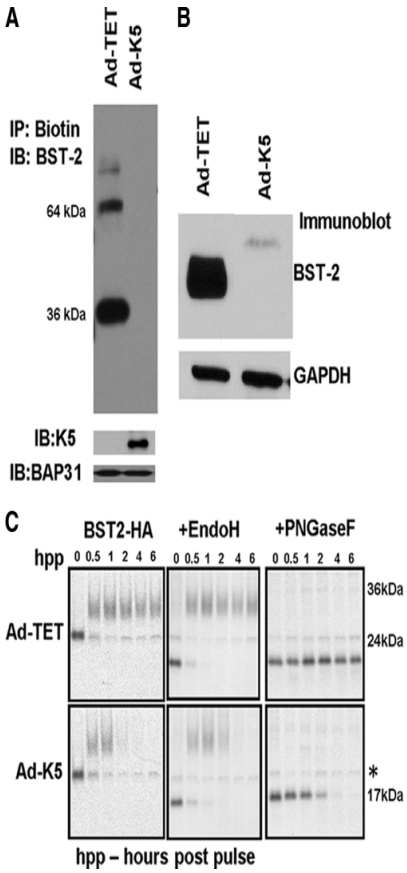

FIG. 2.4.3.

Degradation of BST2 by K5.

(A) BST2 is absent from the cell surface in the presence of K5. E-DMVECs were transduced with Ad-K5 or Ad-TET alone for 24 h, followed by treatment with 500 U/ml IFN-β. After 24 h, cell surface-expressed proteins were biotinylated. Upon cell lysis, biotinylated proteins were captured with avidin and separated by SDS-PAGE. BST2 was visualized by immunoblotting with anti-BST2 (top), anti-K5 (middle), or anti-BAP31 (bottom) as a loading control. (B) K5 reduces steady-state levels of BST2. E-DMVECs were transduced with Ad and treated with IFN-β as described for panel A. Cells were lysed in SDS sample buffer, and the lysate was separated by SDS-PAGE followed by immunoblotting with HM1.24 or anti-GAPDH. (C) Increased degradation of endo H-resistant BST2 by K5. Human fibroblasts stably transfected with the lentivector pCDH-BST2-HA were pulse labeled for 15 min, followed by a chase, as indicated. BST was immunoprecipitated with anti-HA after treatment, followed by treatment with endo H or PNGase F when indicated. SDS-PAGE and autoradiography revealed a 24-kDa glycosylated endo H-sensitive precursor that was converted to an 34-kDa endo H-resistant glycoprotein. Deglycosylated BST2 migrated at about 17 kDa, consistent with its predicted molecular mass of 19 kDa. A nonspecific 24-kDa band comigrated with the 24-kDa form of BST2 (*).

same low molecular mass as the endo H-sensitive form. Since the predicted molecular mass of BST2 is 19.6 kDa, we concluded that the low-molecular-mass form corresponded to deglycosylated BST2. The large shift in molecular mass is consistent with both predicted glycosylation sites being used [83]. The 36-kDa mature protein is relatively long-lived, with a half-life of more than 6 h. In the presence of K5, the synthesis of the endo H-sensitive form of BST2 was unhampered. However, the endo H-resistant form of BST2 was short-lived, with a half-life of less than 2 h. Since endo H resistance indicates that BST2 has acquired modified N-linked glycans in the Golgi apparatus, these results indicate that K5 mediates the degradation of BST2 in a post-ER compartment. Since we were unable to detect BST2 at the cell surface by biotinylation, it further seems that K5 intercepts BST2 en route to the cell surface.

2.4.4 Proteasome inhibitors, but not endosomal inhibitors, prevent BST2 degradation

Post-ER degradation implies that BST2 is targeted for degradation in lysosomes via the multivesicular body (MVB) pathway, as described for several other K5 targets [69]. In such instances, K5-mediated degradation could be inhibited using small-molecule inhibitors of vacuolar ATPases, such as the macrolide ConA [67, 101, 109]. Surprisingly, however, surface expression of BST2 was not restored in the presence of ConA (Fig. 2.4.4A). In contrast, the proteasome inhibitor MG132 completely restored BST2 surface expression (Fig. 2.4.4A). To determine whether proteasomal or endo/lysosomal inhibitors prolonged the half-life of BST2, we performed pulse-chase experiments, with MHC-I molecules as a control. As shown in Fig. 2.4.4B, MG132 efficiently prevented the degradation of both MHC-I molecules and BST2. In contrast, ConA prolonged the half-life of BST2 but ultimately did not prevent its degradation, whereas MHC-I degradation was completely inhibited. This result was unexpected since the proteasome is unable to degrade transmembrane proteins once they have exited the ER. An alternative explanation for this result is that MG132 treatment is known to deplete free ubiquitin

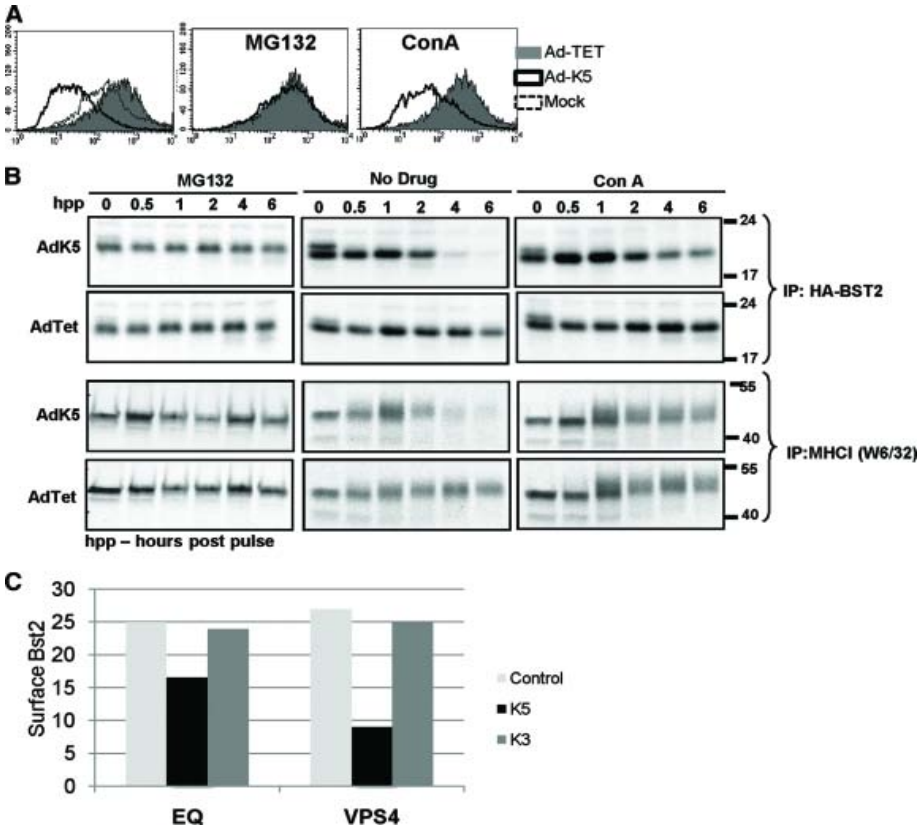

Figure 2.4.4

Proteasomal but not lysosomal inhibitors prevent K5-mediated BST2 degradation.

(A) Proteasome inhibitors restore cell surface expression of BST2 in the presence of K5. Stable transfectants expressing BST2-HA were mock treated (dashed line) or transduced with Ad-K5 (black line) or Ad-TET (gray area) for 24 h, followed by treatment with MG132 (20 μM) or ConA (50 nM) for 12 h. BST2 expression was monitored by flow cytometry using anti-HA. (B) Proteasome inhibitors prevent K5-mediated BST2 degradation. Human fibroblasts stably expressing BST2-HA were metabolically labeled for 15 min, and the label was chased for the indicated times prior to immunoprecipitation and PNGase F treatment. Prior to being labeled, human fibroblasts were pretreated for 12 h with MG132 (20 μM) or left untreated. Immunoprecipitation was done with anti-HA antibody. (C) Overexpression of dominant-negative VPS4-EQ partially restores BST2 surface expression compared to wild-type VPS4 in K5-transfected cells. HeLa cells were cotransfected with control plasmid, K5, or K3 and with wild-type VPS4 or VPS4-EQ. Twenty-four hours later, transfected cells were analyzed by flow cytometry. Surface expression of BST2 on VPS4-expressing cells (as detected by GFP positivity) was measured by flow cytometry using anti-BST2.

from cells, thus affecting ubiquitin-mediated endocytosis [110]. Moreover, proteasome inhibitors were previously shown to prevent sorting of MHC-I to late endosomal complexes of K3-expressing cells [111]. To determine whether BST2 was targeted to lysosomes via the MVB pathway, we cotransfected K5, K3, or a control plasmid with VPS4 or the EQ mutant of VPS4, which inhibits MVB formation [105]. BST2 surface levels, monitored by flow cytometry, were reduced in the presence of VPS4, but to a much lesser extent in the presence of VPS4-EQ (Fig. 2.4.4C). Therefore, we concluded that K5 targets BST2 for lysosomal destruction via the MVB pathway. Why endo/lysosomal targeting of BST2 by K5 was less sensitive to proton pump inhibitors than MHC-I targeting remains to be investigated.

2.4.5 Ubiquitination of cytoplasmic lysines is required for BST2 downregulation

As a ubiquitin ligase, K5 transfers ubiquitin to the cytoplasmic (and usually carboxy-terminal) portion of its substrates [62]. Ubiquitin is conjugated to lysines but can also be transferred to cysteines in the absence of lysines [112]. To determine whether BST2 is ubiquitinated in the presence of K5 and whether this ubiquitination is necessary for BST2 downregulation, we replaced two amino-terminal lysines that are predicted to face the cytosol (K18 and K21) with arginines. The resulting construct, BST2-KR, as well as wild-type BST2, was stably transfected into human fibroblasts. When transfectants were transduced with Ad-K5, only BST2 was significantly downregulated

(Fig. 2.4.5A). In contrast, BST2-KR was largely unaffected. In human fibroblasts stably transfected with an HA-tagged version of BST2-KR, the half-life of BST2 in the presence of K5 was nearly indistinguishable from that in Ad-TET-transduced cells. In contrast, MHC-I molecules immunoprecipitated from the same lysates were turned over more rapidly in Ad-K5- than in Ad-TET-transduced cells. These observations strongly suggested that ubiquitination plays an essential role in BST2 downregulation by KSHV

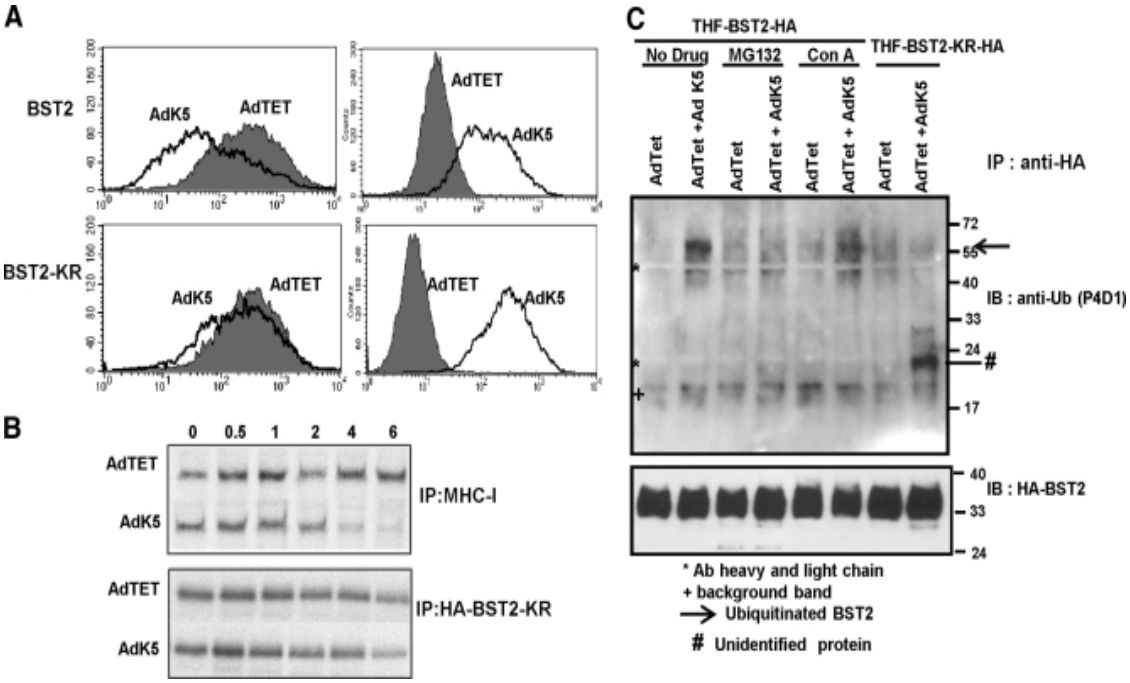

Figure 2.4.5

Ubiquitination of cytoplasmic lysines of BST2 is required for K5-mediated degradation.

(A) Cytoplasmic lysines are required for BST2 downregulation from the cell surface. Flow cytometry was performed with human fibroblasts stably transfected with BST2 or lysine-deleted BST2-KR and transduced with Ad-K5 (black) or Ad-TET (gray) for 24 h. BST2 was detected with the anti-BST2 antibody. (B) Lysine-deleted BST2 is resistant to K5-mediated degradation. BST2-KR-HA-expressing fibroblasts were transduced with Ad-K5 or Ad-TET for 24 h, followed by pulse-chase metabolic labeling and immunoprecipitation with antibodies to MHC-I (W6/32) or anti-HA. Note that the half-life of MHC-I molecules was decreased in the presence of K5, whereas that of BST2-KR-HA was unaffected. (C) BST2 is ubiquitinated in the presence of K5. BST2-HA-expressing fibroblast cells were transduced with Ad-TET alone or with Ad-TET and Ad-K5 in the presence of MG132, ConA, or no drug for 10 h. The cells were harvested, immunoprecipitated using anti-HA antibody, and immunoblotted with anti-ubiquitin antibody (P4D1). Likewise, BST2-KR-HA-expressing fibroblasts were transduced with Ad-TET alone or with Ad-TET and Ad-K5 for 10 h and then immunoprecipitated and immunoblotted. Nonspecific and unidentified protein bands are indicated. Ab, antibody.

K5. To further determine whether the cytosolic lysines were ubiquitinated in the presence of K5, we immunoprecipitated BST2-HA or BST2-KR-HA from Ad-K5- or Ad-TET-transduced stably transfected fibroblasts and probed immunoblots with ubiquitin-specific antibodies. As shown in Fig. 2.4.5C, several high-molecular-weight bands appeared in immunoblots for Ad-K5-transduced fibroblasts. These ubiquitin-reactive bands were absent for Ad-TET- or Ad-K5-transduced BST2-KR-HA-expressing cells. We concluded that they represent oligo-ubiquitinated BST2 and that ubiquitination takes place at the intracellular lysines. Interestingly, a low-molecular-weight ubiquitinated protein was immunoprecipitated with BST2-KR-HA in the presence of K5. The identity of this protein is currently unknown, but it might represent a protein that is ubiquitinated in a complex with K5 and BST-KR. We further examined the effect of MG132 on BST2 ubiquitination. As shown in Fig. 2.4.5C, ubiquitinated BST2 was not detected in the presence of MG132, consistent with MG132 inhibiting ubiquitination of BST2. In contrast, ConA did not affect ubiquitination of BST2 and did not stabilize a ubiquitinated intermediate as observed previously [67, 101]. We therefore concluded that MG132 restored BST2 surface expression by depleting free ubiquitin and thus preventing K5 from ubiquitinating BST2.

2.4.6 BST2 reduces KSHV recovery

To determine whether BST2 affects the production of KSHV upon reactivation, we infected HeLa cells, which express high levels of endogenous BST2 (4), with rKSHV.219, a recombinant virus that expresses GFP under the control of a constitutive promoter and red fluorescent protein under the control of a lytic promoter [103]. rKSHV.219 established latent infection in HeLa cells upon puromycin selection, as described previously [103]. Latently infected cells were treated with siRNA to K5 or control siRNA, and virus was reactivated with Ad-RTA together with sodium butyrate. Virus release into the supernatant was monitored by transferring the supernatant to HEK293 cells and monitoring GFP fluorescence. As shown in Fig.

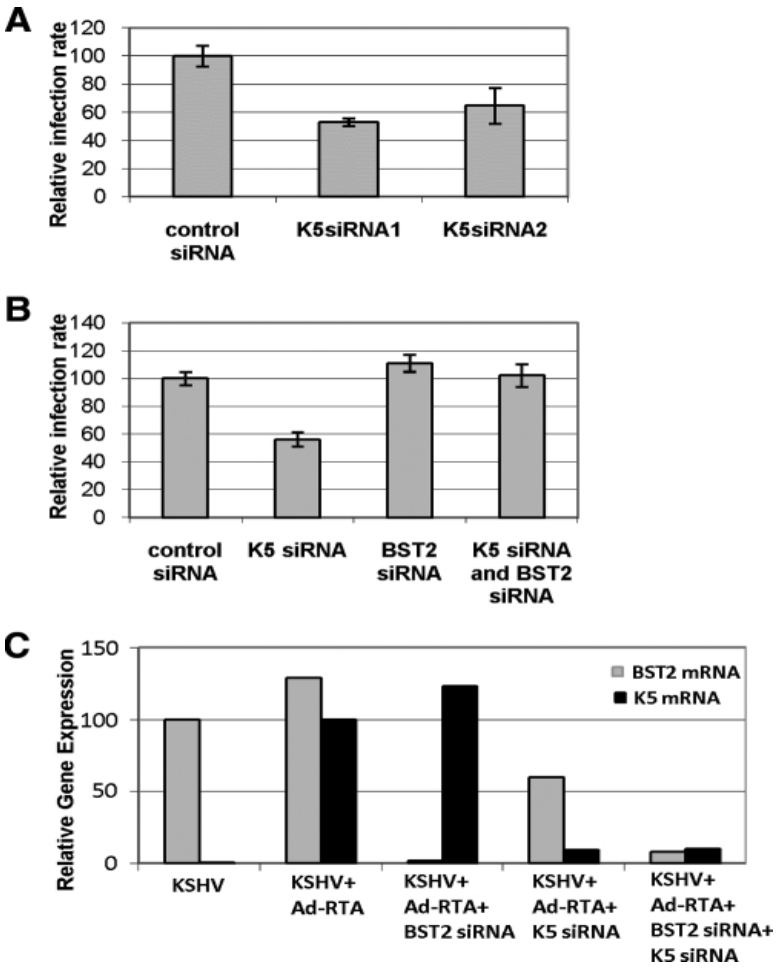

FIG. 2.4.6.

KSHV K5 facilitates viral release by removing BST2 from HeLa cell surface.

(A) Decreased viral release in the presence of K5 siRNA. HeLa cells infected with rKSHV.219 were transfected in triplicate with two K5 siRNAs or control siRNA. The transfections were repeated in 8 h, and 24 h thereafter, Ad-RTA was added to reactivate the virus. After an additional 48 h, cell supernatants were harvested, filtered, and used to infect 293 cells. Forty-eight hours later, GFP fluorescence was monitored in 293 cells by flow cytometry. (B) BST2 siRNA restores KSHV release in the absence of K5. HeLa cells infected with KSHV were transfected with the indicated siRNAs as described for panel A. (C) BST2 and K5 transcript levels upon siRNA treatment. qPCR analysis was performed to determine the relative amounts of BST2 and K5 transcripts in siRNA-transfected HeLa cells infected with KSHV. The K5 transcript level in Ad-RTA-induced KSHV-infected HeLa cells was set to 100%, whereas the BST2 level was set to 100% in the nonactivated KSHV-infected HeLa cells.

2.4.6A, we observed a 50% reduction of KSHV recovery from HeLa cells in the presence of K5 siRNA. This reduction of KSHV release was due to BST2, since KSHV recovery was restored to control levels when K5 siRNA was cotransfected with siRNA to BST2 prior to reactivation (Fig. 2.4.6B). Cotransfection of the two siRNAs did not affect their ability to reduce target transcript expression (Fig. 2.4.6C). We interpret these results as an indication that BST2 interferes with KSHV release.

2.5 Discussion

Our data strongly suggest that despite its unusual topology, BST2 is a bona fide target of K5. As such, K5 mediates the ubiquitination of one or both cytoplasmic lysines at a post-ER location in the cell. Previously, it was demonstrated that BST2 continuously recycles between the plasma membrane and the trans-Golgi network (TGN) [77]. In contrast to other K5 targets that are internalized from the cell surface [70, 101], we were unable to detect BST2 at the plasma membrane, even upon biotinylation (Fig. 2.4.3A). Thus, K5 most likely intercepts IFN-induced BST2 in the TGN. This conclusion is also supported by the observation that K5 does not degrade BST2 prior to ER exit.

Given the post-ER degradation of BST2, the most likely destination of ubiquitinated BST2 is the lysosome. Surprisingly, however, inhibitors of lysosomal acidification did not prevent K5-mediated BST2 degradation. This is in contrast to the case for all other K5 targets (with the notable exception of newly synthesized CD31 [31] studied to date (reviewed in reference 40). In each case tested, vacuolar ATPase inhibitors restored surface expression of the respective K5 targets. Moreover, in several instances we observed that ConA stabilized a ubiquitinated intermediate in the presence of K5 or other viral MARCH proteins [67, 101, 109]. The failure of ConA to prevent K5-mediated BST2 degradation could thus be interpreted as evidence that BST2 is not degraded by the lysosome. Indeed, inhibitors of the proteasome, the second major proteolytic system in the cell, prevented BST2 degradation and restored BST2 surface expression. However, we consider it unlikely that the proteasome is directly involved in degrading BST2 for several reasons, as follows. (i) Proteasomal degradation of transmembrane proteins requires their retrograde translocation to the cytosol [113], a process that is confined to ER-localized transmembrane proteins. Since BST2 acquired endo H resistance prior to K5-mediated turnover, proteasomal degradation would thus require retrograde transport of BST2 to the ER, for which there is currently no evidence. (ii) We did not observe a degradation intermediate of BST2 in the presence of proteasome inhibitors. In contrast, cytosolic, deglycosylated, and ubiquitinated

intermediates have been reported for MHC-I molecules degraded by the proteasome in the presence of human cytomegalovirus US2 and US11 [114]. (iii) Proteasomal degradation generally requires polyubiquitination via a lysine 48 linkage [115]. In contrast, we observed only a few discreet ubiquitinated bands for BST2, consistent with a model of oligo-ubiquitination via lysine 63 linkage proposed for K3 [68]. Mono-ubiquitination at single or multiple cytoplasmic lysine residues as well as K63-linked oligo-ubiquitination has been implicated in endocytosis of proteins destined for lysosomal degradation via the MVB pathway [116-119]. (iv) Inhibition of MVB formation by a dominant-negative form of VPS4 restored BST2 surface expression. Taking all these observations together, we therefore consider it unlikely that BST2 is extracted to the cytosol and destroyed by the proteasome.

A more likely explanation is that MG132 limits the ability of K5 to ubiquitinate BST2, since prolonged treatment with proteasome inhibitors depletes the cellular pool of free ubiquitin [120]. This is supported by the observation that ubiquitinated BST2 was no longer observed in MG132-treated cells, despite the presence of K5. Thus, the very first step of ubiquitin-mediated mistargeting of BST2 was inhibited by MG132. Similarly, it was previously concluded that proteasome inhibitors prevent early-to-late endosomal sorting of MHC-I molecules in the presence of KSHV K3 [111]. It is thus conceivable that ubiquitin depletion prevents the first step in TGN-to-lysosome mistargeting of BST2. Why this process is resistant to macrolides, such as ConA, that normally block the transport of cargo from late endosomes to lysosomes [121], remains to be investigated.

It is possible that the unique topology and GPI linkage of BST2 contribute to this unusual degradation pathway. The addition of a GPI linker generally renders proteins resistant to ubiquitination due to the concomitant removal of cytoplasmic domains that can be targeted for ubiquitination. To our knowledge, BST2 is the first reported case of a GPI-linked protein that also carries a cytoplasmic domain that is susceptible to ubiquitination. Similar to most GPI-linked proteins localized to cholesterol-rich

membrane microdomains, BST2 was shown to localize to lipid rafts [77]. Given its unique topology, it was hypothesized that BST2 lines the borders of lipid rafts like a fence, with the N terminus located in the nonraft portion of the membrane. It will be interesting to determine how K5-mediated ubiquitination affects the partitioning of BST2 to lipid rafts and whether other proteins that colocalize with BST2 at lipid rafts are affected by BST2 ubiquitination. For example, ubiquitination could result in the extraction of BST2 from the raft, or it could trigger the mistargeting of other raft-associated proteins. Thus, BST2 ubiquitination by K5 is a unique model with which to study the ubiquitin-mediated sorting of a raft-associated protein.

BST2 downregulation correlated with K5 expression in KSHV-infected ECs. Upon primary infection, K5 is transiently expressed prior to the establishment of a restricted latency-associated expression pattern. Consistent with K5 shutoff during latency, BST2 induction by IFN was not inhibited by K5 in latently infected cells. Instead, basal levels of BST2 were elevated in latently infected cells (Fig. 2.4.2), suggesting a low-level activation of BST2 expression in virally infected cells. Given that multiple stimuli (type I and type II IFNs, as well as TNF-) induce BST2 expression, we interpret this as evidence that infection by KSHV upregulates the transcription of BST2. This IFN-independent, virus-dependent BST2 induction could be due to activation of NF-B by the latency-associated transcript vFLIP [122]. In fact, it was recently reported that human umbilical ECs transduced with vFLIP showed a severalfold upregulation of the BST2 transcript [123]. Alternatively, viral DNA could activate innate pattern recognition receptors, such as the recently identified IFN-activating repeat element in 2-herpesvirus genomes [124]. In vivo, BST2 induction might thus result from local IFN secretion by neighboring infected cells or infiltrating lymphocytes as well as from activation of the innate immune response in infected cells.

The targeted elimination of BST2 by K5 is thus part of KSHV's countermeasures against the host IFN response. Several gene products of KSHV are known to interfere at

different steps of the IFN cascade, including (i) the signaling pathways that lead to the induction of the IFN gene, (ii) the signaling cascade triggered by IFN binding to its cellular receptors, and (iii) counteraction of the antiviral activity of IFN-stimulated genes (ISGs). For example, viral IFN regulatory factors (vIRFs) disrupt the IFN-independent activation of host IRFs [108]. Moreover, IFN--dependent signaling can be counteracted by K3 and K5, which both downregulate the IFN- receptor [125], and type I IFN-dependent signal transduction was shown to be inhibited by vIRF2 [126] and by the RIF protein encoded by ORF10 [127]. However, we observed only a slight reduction of BST2 mRNA induction by IFN-β in Ad-RTA-transduced versus control-transduced cells (Fig. 2.4.2B), suggesting that any inhibition of signal transduction from the type I IFN receptor by reactivated KSHV was unable to substantially interfere with BST2 induction. The downregulation of BST2 by K5 thus belongs in the category of viral gene products preventing the antiviral action of ISGs. While counteracting ISG function has been described for other viruses [128], very little is known about such ISG countermeasures by KSHV. One example is the inhibition of IRF7, an IFN-induced transcription factor, by ORF45 and vIRF3 [49, 129]. Thus, the K5-mediated degradation of BST2 is one of the first examples of KSHV directly destroying an IFN-induced protein.

The efficient inhibition of IFN-induced BST2 expression by K5 implies that K5 might play a role in counteracting an innate immune mechanism. One possibility is that in the absence of K5, BST2 prevents the release of KSHV from the host cell, similar to the case reported for VPU-deleted HIV-1. This hypothesis is supported by our observation that KSHV release from HeLa cells was decreased in the presence of K5 siRNA but restored when BST2 expression was also inhibited. Herpesviral egress is quite different from that of retroviruses and filoviruses, as it involves the envelopment of tegument-covered viral capsids in the TGN followed by exocytosis of virion particles by vesicular transport [130]. Thus, it seems likely that BST2 interferes with this process in the TGN. Recent observations further suggest that BST2 inhibition of HIV-1 is confined to certain cell types, e.g., macrophages and HeLa cells, but is less efficient in other cell

types, e.g., T cells [96]. Since experiments with K5 siRNA in other cell types have so far been inconclusive (data not shown), it is possible that an antiviral activity of BST2 is cell type specific. Since KSHV is capable of infecting a number of cell types, with B cells and ECs being the cells most commonly infected in vivo, further work will be required to determine the effect of BST2 on KSHV propagation in the absence of K5.

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Chapter 3

Kaposi's Sarcoma-associated Herpesvirus Egress is Reduced by BST-2

3.1 Introduction

KSHV employs a wide variety of methods to evade the immune system of host cells. One of these mechanisms is the viral ubiquitin E3 ligase, K5, also known as modulator immune response 2 [62]. K5 has been previously shown to downregulate MHC class I, thereby preventing elimination of the infected cell by cytotoxic T lymphocytes [70]. An effort to identify additional targets of K5 found BST-2 to be downregulated in the presence of K5 [74]. This result followed the recent identification of BST-2 as having the capability of tethering nascent virions to the cell surface [85]. This relationship is quite interesting and as such we sought to further clarify this process.

After providing evidence that K5 down regulates BST-2, and that BST-2 does indeed inhibit viral egress, we sought to further elucidate this process of downregulation. We were concerned that some of our results might be due to an artifact of working in HeLa cells, so other cell types were utilized to confirm that our results indeed represented a true mechanism. Again, siRNA knockdown of K5 was performed on both 293T that were expressing BST-2 to examine the differences in the amount of KSHV produced.

3.2 Methods

3.2.1 Stable BST-2 Expressing Cell Lines

In order to generate 293 T cells that stably express BST-2, lentiviral vector pCDH-CMV-MCS-EF1-Puro (System Biosciences, Mountain View, CA) was used. First *bst*-2 cDNA was amplified as an NheI/BamHI fragment by PCR using the Pfu enzyme (Stratagene, San Diego, CA), and then inserted into the lentiviral vector. A hemagglutinin (HA) tag was inserted at position 146 of BST-2. This construct along with pHP-dl-N/A packaging construct, the pHEF-VSVG envelope construct were triple-transfected into 293Ts (both constructs were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Lung-Ji Chang) [131]using Effectine (QIAgen, Germantown, MD) at a ratio of 3:6:1. 48 hours after transfection the supernatants were harvested and filtered with a 0.8μm filter. The lentiviral BST-2 HA146 construct, along with an empty vector control, was used to generate the stable cell lines in 293T cells. Vero cells expressing BST-2 were generated in a similar manner, however, the HA tag on this BST-2 construct was located at position 110 rather than 146. Cell lines were maintained under puromycin selection (Invitrogen, Carlsbad, CA) at 0.5μg/ml. BST-2 expression was verified through fluorescent associated cell sorting (FACS) using an HA-specific antibody (Covance, Princeton, New Jersey).

3.2.2 rKSHV .219

The virus used for these experiments was rKSHV.219 (kindly provided by J. Vieira). rKSHV.219 expresses green fluorescent protein (GFP) under the control of a constitutive promoter and red fluorescent protein (RFP) under the control of a lytic promoter and can be maintained under puromcyin selection [103].

3.2.3 siRNA

Two different K5 siRNAs smart pools and a GAPDH smart pool were used from Dharmacon (Dharmacon/Thermo Scientific, Rockford IL).

3.2.4 K5 Knockdown in 293 BST-2 HA146

BST-2 HA146 293 cells were seeded in a 24-well format at a density of $1.25x10^5$. 24 hours later the cells were transfected with K5 siRNA, and 24 hours post-transfection the cells were infected with rKSHV.219 in the presence of 1.2µl polybrene. Another 24 hours later the cells were split 1 to 3, and the next day lytic infection was induced by transfection of RTA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer's protocol, with the addition of 12.5 µM sodium butyrate two hours after the transfection. After two more days the cells were examined for red positive cells by fluorescent microscopy and supernatant was harvested following verification of positive cells. The supernatant was filtered with a 0.8µm filter and applied to 293T cells (seeded the previous day at $1.25x10^5$) in the presence of polybrene. Two days following this infection, FACS was done to measure the percentage of KSHV GFP positive cells

3.2.5 Vero BST-2 HA110 Growth Curves

Vero BST2 HA110 and Vero pCDH cells were seeded in a 12 well format at 5x104 per well. The following day, one set of cells were infected with AdRTA at an MOI of 20 so lytic infection would occur upon primary infection, cells not to be infected with adRTA had the media changed. Twentyfour hours after AdRTA all cells were infected with rKSHV.219 with polybrene. Cells were harvested at 24, 48 and 72 hours post infection. FACS was performed to determine the amount of GFP positive cells.

3.3 Results

3.3.1 K5 Knockdown in 293 BST-2 HA146

To determine whether BST-2 interfered with KSHV egress we examined infections normal 293T cells and stably transfected 293 BST-2HA146. In order to examine the effects of K5 on restoring viral egress K5 siRNA knockdowns were preformed. Since we know that BST-2 is a target for downregulation by K5, then it can be presumed that in cells that express BST-2 that K5 has been knocked down in, that there would be a limited amount of viral egress, due to BST-2 not being downregulated. However, in cells that do not express BST-2 it would be expected that the amount of KSHV released from the cell would not differ whether K5 had been knocked down or not.

In 293T cells, which do not express BST-2, the amount of KSHV released is not changed by the K5 siRNA knockdown. However in the 293 BST-2HA146 cells the presence of KSHV in the supernatant is reduced by approximately fifty percent when K5 is knocked down (Figure 3.3.1). This leads us to believe that BST-2 is the factor that is limiting the viral release and that K5 is necessary to downregulate BST-2.

3.3.2 Vero BST-2 HA110 Growth Curves

We had noted in the siRNA knockdowns that in cells expressing high amounts of BST-2 verses cells not expressing BST-2 there was a difference in the amount of KSHV found in the supernatant of infected cells. In order to determine whether this was an effect of different conditions such as the way cells were growing, we sought to determine if the amount of KSHV released by cells either expressing BST-2 or not was an actual effect of the presence of BST-2. The rate at which infection spread was measured in Vero BST-2 HA110 and Vero pCDH cells that were undergoing lytic infection. This rate

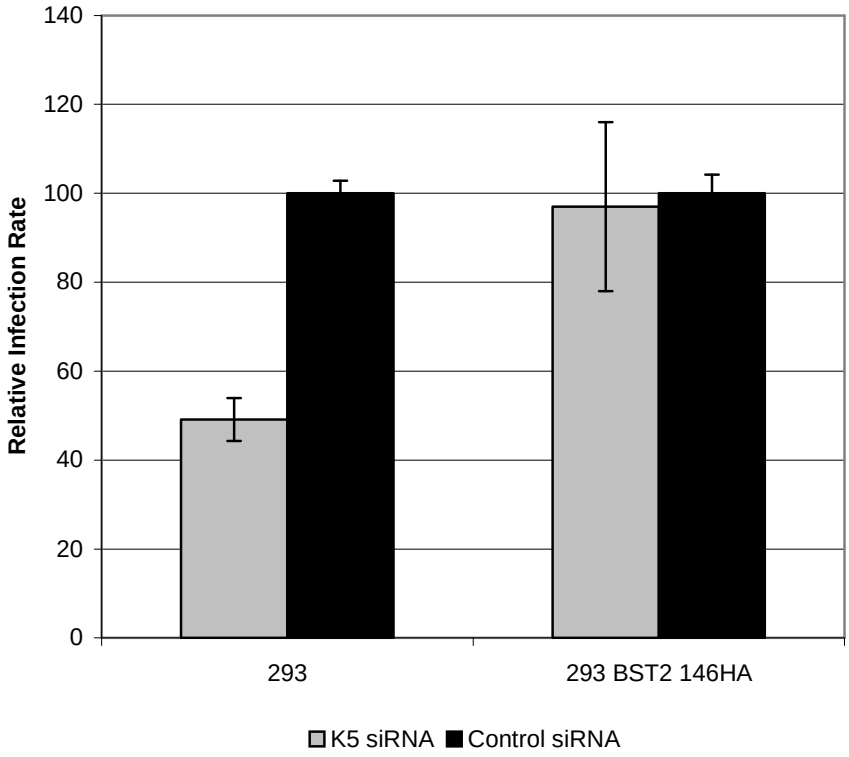

Figure 3.3.1

K5 knockdown reduces virus released in 293T cells expressing BST-2.

KSHV viral egress was measured by presence of virus in the supernatant of these cells as shown by the amount of GFP positive cells in FACS. Cells transfected with control siRNA were set to one hundred percent to represent the normal amount of virus being released from the cells, and compared to the amount in K5 knockdowns. These were compared to the difference in KSHV in the supernatant of cells that did not express BST-2.

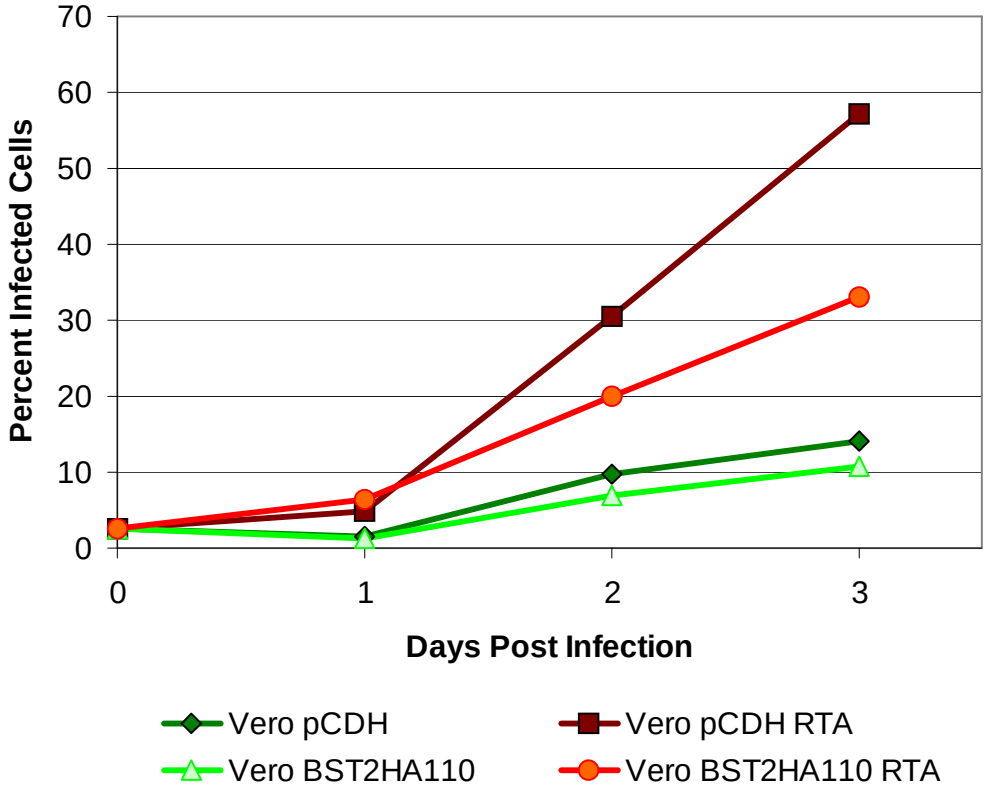

Figure 3.3.2

BST-2 Restricts spread of KSHV

KSHV infection was monitored over three days by the percent GFP positive cells detected by FACS. Both latent (green) and lytic (red) cells were tracked for the rate at which infection spread. These could be compared between the two cell lines, Vero pCDH (dark) and Vero BST-2HA110 (light).

was compared against the spread of infection in latenly infected cells in order to confirm that any differences were due to virus produced, not factors such as rate of cell growth.

In latently infected cells not expressing BST-2 (Vero pCDH) the spread of KSHV was approximately 1.3 more than cells expressing BST-2 (Vero BST-2HA110). However, upon induction of lytic infection, that rate at which infection spread in Vero pCDH was about twice that of Vero BST-2. This would indicate that while the cells might have been growing slightly slower, that difference is not enough to allow for the rate differences of infection spread. This leads us to believe that BST-2 is causing less virus to be released from the cells to go on infect the other cells in that culture.

3.4 Conclusions

We have previously demonstrated that K5 is capable of downregulating BST-2 in HeLa cells. This was shown by K5 siRNA and BST-2 knockdown; when K5 is knocked down, less KSHV is released from the cells. However, when K5 and BST-2 are both knocked down the amount of KSHV released returns to normal, suggesting that BST-2 is responsible for inhibiting viral egress, and that K5 is targeting BST-2 for downregulation.

We observed that in cells not expressing BST-2, K5 could be knocked down with no ill effect on the amount of virus released from the cells. However, in cells that express BST-2, KSHV viral egress was reduced by about fifty percent when K5 levels are lowered by siRNA treatment. This led us to conclude that our previous observations that K5 targets BST-2 for down regulation and that BST-2 inhibits viral egress are indeed correct and not just an artifact of working in HeLa cells.

Whether the expression of BST-2 was affecting the amount of virus released over time during lytic infection was also examined. By comparing the rates of spread of infection in Vero pCDH versus Vero BST-2HA110 during both latent and lytic infection we determined several things. First the initial infection in either cells was very similar, suggesting that BST-2 was not interfering with entry. We also identified that while the different cells did not grow at exactly the same rate, that the rate at which infection spread was quite different. By taking what we know of the nature of BST-2 inhibiting viral egress, the evidence that infection is spreading about half as fast in the BST-2 cells leads us to believe that the reason for this is because of limited release of the virus.

Chapter 4

4.1 Discussion

The human herpesvirus KSHV is known to have many immune evasion methods [39]. Among those are the modulators of immune response, ubiquitin E3 ligases K5 and K3. K5 had been known to downmodulate MHC class I in order to help avoid detection by T-cells [70]. When additional targets for downregulation by K5 were sought out by our group, one identified BST-2 [74]. BST-2 has been identified as having the capacity to inhibit viral egress of a number of viruses by retaining them on the cell surface [85, 88]. Taking this information together, establishing the relationship between K5 and BST-2 is quite intriguing, here we further elucidate the interactions of K5 and BST-2.

The data described herein verifies that BST-2 is ubiquitinated by K5 and is subsequently degraded by the lysosome. We further demonstrate that BST-2 is capable of inhibiting viral egress and that normal levels of egress can be restored by K5, thus showing that K5 is responsible for downregulating BST-2.

We sought to establish that BST-2 was downregulated in DMVECs during de novo infection of KSHV (Fig. 2.4.1B). By inducing BST-2 expression with interferon, we demonstrated that KSHV infection had the capability to reduce the amount of detectable BST-2. However, if cells were treated with K5 siRNA BST-2 levels returned to normal. This verifies that K5 is what is responsible for the downregulation of BST-2.

This was further confirmed through latently infected cells being reactivated by RTA to lytic infection (Fig.2.5.2A). Additionally it was shown that downregulation of BST-2 occurs by protein degradation, and not through inhibition of mRNA synthesis. (Fig. 2.4.2B).

We examined the effects of siRNA knockdown of K5, BST-2, or K5 and BST-2 in HeLa cells, which express BST-2, latently infected with KSHV. Upon siRNA knockdown of K5, measured KSHV viral egress was reduced by approximately fifty percent. However, when both K5 and BST-2 are knocked down, KSHV release was restored to normal (Fig. 2.4.6B). In order to show that this was not exclusive to HeLas, this observation was verified in 293Ts that were stably expressing BST-2. When K5 siRNA knockdown was done in BST-2-expressing 293Ts, we again saw that KSHV release was reduced by approximately fifty percent. In contrast, in normal 293Ts that did not express BST-2, K5 siRNA knockdown had no effect on the amount of KSHV released (Fig. 3.3.1). These data demonstrate that K5 targets BST-2 for downregulation, and that successful downregulation of BST-2 is required for full-scale viral egress. We also show that the rate at which lytic KSHV infection spreads throughout cells is greatly reduced in cells that over-express BST-2 compared to those that do not express BST-2 (Fig. 3.3.2). This provides evidence that the BST-2 present in those cells had the capability of reducing the amount of virus released to go on and infect more cells and that there is a point when K5 is not capable of fully downregulating BST-2.

The manner of degradation of BST-2 was also examined. By surface biotinylation it was determined that in the presence of K5, BST-2 was not found on the surface of cells (Fig. 2.4.3A). The point at which BST-2 is downregulated was determined by treating cells with endoglycosidase H (Endo H). Proteins that are post-translationally modified by N-linked glycans are sensitive to Endo H until they progress through the Golgi, after which the mature, cell-surface destined form becomes Endo H resistant. (Fig. 2.4.3C). We showed that K5 downregulates BST-2 that is Endo H

resistant, this suggests lysosomal degradation. In order to further examine the destination of BST-2 we used inhibitors of both lysosomal and proteasomal. Surprisingly, the proteasome inhibitors had the ability to restore surface expression of BST-2 (Fig. 2.4.4A, Fig. 2.4.4B). However, the defined band size would indicate lys63 linked ubiquitination. The possibility of the proteasome inhibitor creating a shortage of ubiquitin could be the reason this was observed. We were able to verify that the lysines of BST-2 were being ubiquitinated using a lysine to arginine mutant that was not susceptible to downregulation by K5 (BST-2 K18R) (Fig. 2.4.5A, Fig. 2.4.5B). Using this construct, we demonstrated that when BST-2 or BST-2K18R was expressed in the presence of K5, only BST-2 is detectable by an ubiquitin antibody (Fig. 2.4.5C).

Recently Pardiue and colleagues have published some data that confirms a great deal of the data shown herein. In findings similar to ours, they showed that K5 is able to downregulate BST-2 on the surface of cells. In addition they showed that the RING domain of K5 was necessary to downregulate BST-2. K5 shRNA knockdown was done in HeLas and like we saw with our siRNA knockdowns of K5, they found that the amount of KSHV released was decreased greatly. They demonstrated that K5 ubquitinates BST-2 and it is degraded in the lysosome, in order to do so, the cytosolic lysines of BST-2 are required. The effect of increasing amounts of BST-2 on the amount of KSHV released was also shown, with more BST-2 there is a greater reduction in viral release. In addition to providing data that supports our findings they also were able to demonstrate that K5 could take the place of Vpu in HIV-1 release [132].

All of these data together helps to further clarify the effect of BST-2 on viral egress. Previously, the viruses that have been shown to be affected by BST-2 are nearly exclusively retroviruses. For those that are not retroviruses, the data provided has been for viral like particles of the filoviruses, Ebola and Marburg and an arenavirus, Lassa [85, 88-91]. They are all RNA viruses. In showing that BST-2 has an effect on viral egress of

KSHV, a DNA virus, it can be suggested that this could be a mechanism that is in place for a wide variety of enveloped viruses.

In addition to showing that BST-2 can inhibit release of different types of enveloped viruses, our data suggests that BST-2 is able to act on viruses that employ different methods of viral egress. Herpesviruses egress is not as simple as budding through the cell membrane; virions bud into secretory vesicles through the endosomal sorting complexes required for transport (ESCRT) pathway [130]. When considered with BST-2 being present in intracellular compartments, the possibility that BST-2 is acting at a point other than at the cell surface seems likely [77]. However, since most have shown BST-2 to gather nascent virions on the cell surface, it would be interesting to further resolve the mechanism in which BST-2 is acting to reduce viral egress in the manner employed by KSHV, and also the fate of the virions.

By applying the further understanding we have gained of the viruses which BST-2 is able to inhibit viral egress, we can expand on the idea of mechanisms of immune evasion. By establishing that BST-2 is able to have an effect of viral egress we raise the question of how successful release can occur with different viruses. Here we have shown that with KSHV the viral egress despite BST2 expression is supported by K5. This further shows the importance of K5's role in the KSHV infection and as an immune modulation mechanism. K5 was known to remodel endothelial cell function, and to contribute to evasion of cellular immune response, and now in addition to that, we see K5 is important for regulating the infected cell's innate immune response.

These data further elucidate the relationship of K5 and BST-2, suggesting that BST-2 plays an important role in innate immunity, and that K5 is quite valuable to KSHV as an immune evasion protein. We demonstrated that in the presence of K5, the amount of BST-2 that is detectable on the cell surface is greatly reduced. In addition, we show that the lysines in BST-2 are required for ubiquitination. We have established that even a

partial knockdown of K5 in cells expressing BST-2 results in a significant decrease in the amount of KSHV released from the cells. We also showed that cells over-expressing BST-2 have the capability to greatly slow down the spread of the infection. From all of this, we can mainly conclude two things: BST-2 is responsible for reduced KSHV viral egress, and BST-2 is a bona fide target for down regulation by K5. Therefore we can deduce that without K5, KSHV would not be successfully released from cells that express BST-2. Ultimately, this is one step in further unveiling the complex interplay between the extensive immune response of the host and the viral countermeasures.

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Biographical Sketch

Jennie Lyn Hines was born in Eugene Oregon on July 7th 1983 to Jeff and Cindy Hines. Jennie and her older brother David grew up in Southeast Portland and attended David Douglas High School. While there, Jennie participated in Honor Society, Science Club, Track and Field and Cross Country. She graduated in 2001 as valedictorian and due to advanced placement classes had earned 79 college credits.

Jennie began study of biochemistry and biophysics as a Presidential Scholar at Oregon State University in 2001. While at OSU she had her first experience in research in the Plant and Pathology department as a Howard Hughes Medical Institute student. Jennie graduated in just three years in 2004.

After graduating from OSU Jennie began working at OHSU in Medical and Molecular Genetics on a project involving Fanconi Anemia and double strand DNA break repair. While there she participated on a project that resulted in her first publication in the *Journal of Biological Chemistry*. After a year and a half Jennie moved back to Corvallis and took a job in Pharmacy at OSU where she set up a new lab. She then moved to Siga Technologies and joined the hemorrhagic fever virus group. While there Jennie worked to develop antivirals for a variety of viruses. After two years at Siga, she decided to continue her education and enrolled at OGI to begin work on a masters in biochemistry and molecular biology. While taking classes as a part time student, Jennie began working at the Vaccine and Gene Therapy Institute in the lab of Klaus Frueh. After a year there she became a full time student and applied the research she was doing in the Frueh lab towards her thesis, a large part of which was published in the *Journal of Virology*.

Jennie currently resides in Hillsboro with her fiancée Dan Womack and their dog Kitty. After graduation she plans to stay at VGTI and join the lab of Michael Jarvis to work on vaccines using RhCMV.