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The role of the structural domains of human BST-2 in inhibiting the release of xenotropic murine leukemia virus-related virus

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ABSTRACT

BST-2 (bone marrow stromal cell antigen 2) is an interferon-inducible protein that inhibits the release of a variety of enveloped viruses by tethering viral particles to the cell surface. Xenotropic murine leukemia virus-related virus (XMRV) is a gamma-retrovirus that was derived from the recombination of two endogenous murine leukemia viruses during the production of a prostate cell line in mice. In this study, we observed that XMRV was highly sensitive to the inhibition by human BST-2. We were able to determine the structural domains of BST-2 that are essential to restrict XMRV, including the transmembrane domain, the coiled-coil ectodomain, the C-terminal glycosylphosphatidylinositol (GPI) anchor, the two putative N-linked glycosylation sites, and the three extracellular cysteine residues. Protease treatment effectively released XMRV particles into the supernatant, supporting the notion that BST-2 tethered nascent particles to the cell surface. These data suggest that BST-2 poses a strong restriction toward XMRV production.

1. Introduction

Host restriction factors often determine the tissue tropism of a virus and limit virus cross-species transmission [1,2]. One such restriction factor is BST-2 (also named tetherin, CD317 or HM1.24), which was discovered through its ability to inhibit the release of human immunodeficiency virus type 1 (HIV-1) [3,4]. BST-2 is a homodimeric, lipid raft-associated type II integral membrane glycoprotein. BST-2 contains an N-terminal cytoplasmic tail (CT), a transmembrane domain (TM), a coiled-coiled ectodomain, two potential N-linked glycosylation sites, and a C-terminal glycosylphosphatidylinositol (GPI) anchor [5,6]. The antiviral function of BST-2 is attributed to its unique two-membrane-anchor topology. Since the discovery of its anti-HIV-1 activity, BST-2 has been shown to restrict the release of many enveloped viruses, including HIV-2, simian immunodeficiency virus (SIV), Kaposi's sarcoma herpes virus (KSHV), Lassa virus, Marburg virus, and Ebola virus [7–10]. The importance of BST-2 in host antiviral defense is demonstrated by the study showing that BST-2 knockout mice exhibited severe pathology upon infection by murine leukemia virus (MLV) [11].

Xenotropic murine leukemia virus-related virus (XMRV) was originally reported in prostate cancer tissues from patients with a low-activity variant of RNase L [12,13]. This finding has been questioned by subsequent studies [14]. XMRV has also been linked to chronic fatigue syndrome (CFS) [15], but this observation could not be reproduced in other studies [16,17]. Results of several groups argued that the XMRV DNA detected in human clinical samples resulted from the contamination by mouse DNA containing an MLV-like sequence [18–22]. XMRV was traced back to the recombination of two endogenous MLVs, PreXMRV-1 and PreXMRV-2, during *in vivo* tumor passaging in nude mice [23,24]. These data led to the retraction of the papers by Lombardi et al. and Lo et al. [25–27]. It is now well received that XMRV does not circulate in the general human population [28].

Although the association of XMRV with human diseases now appears unlikely, XMRV can still replicate in human cell lines *in vitro* [29]. This provides an opportunity to study how XMRV responds to host restriction in human cells. Indeed, studies have shown that XMRV infection is inhibited by human APOBEC3G and human BST-2 [30]. Here, we confirmed and extended the observation that XMRV is sensitive to the inhibition by BST-2 [30]. We further showed that human BST-2 was sufficient to tether nascent XMRV particles to the cell surface. We also used mutagenesis analysis to characterize the features of BST-2 that are required for its anti-XMRV activity.

2. Materials and methods

2.1. Plasmid DNA and antibodies

The human BST-2 (hBST-2) cDNA was a gift from Chen Liang (Lady Davis Institute for Medical Research, McGill University).

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The pcDNA-BST2-HA construct expresses internally HA-tagged BST-2 proteins (with the HA tag inserted immediately 3′ to BST-2 codon 154). Single amino acid mutations at BST-2 cysteine codons and glycosylation sites were generated by PCR-based mutagenesis. The BST-2-HA mutants delCC, delGPI and delTM were generated according to methods that were described previously [31]. The proviral DNA construct for XMRV was provided by Stephen Goff (Columbia University). The HA.11 antibody was purchased from Covance (MMS-101P). The goat anti-MuLV CA p30 antibody was provided by Stephen Goff (Columbia University). The Vpu and hBST-2 antisera were obtained from the NIH AIDS Research and Reference Reagent Program. PEI (408727), subtilisin (P5380), PI-PLC and TPCK-treated trypsin (T1426) were purchased from Sigma.

2.2. Cell culture and virus production

Human embryonic kidney (HEK293T, ATCC #CRL-11268), HeLa (ATCC #CCL-2) and Vero (ATCC #CCL-81) cells were cultured in complete DMEM supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Vero cells (African green monkey kidney epithelial cells) were transfected with pcDNABST-2-HA DNA, and grown in medium containing G418 (400 μg/ml) to select for stably transfected cells. The pcDNA3.1 vector (Invitrogen) was used to generate the control cell line.

To produce viruses, cells were seeded in 10-cm dishes (5×10^6 - cells per dish) 24 h prior to transfection. The proviral DNA and other plasmids were transfected using PEI (Sigma) according to the manufacturer's instructions. Forty-eight hours after transfection, the culture supernatants were filtered through a 0.45 μm filter, and virus particles were pelleted by ultracentrifugation through a 20% sucrose cushion at 35,000 rpm for 1 h at 4 °C using a Beckman SW 41 rotor.

2.3. Western blotting

Cells or viruses were lysed in RIPA buffer (0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA), and equal amounts of cell or viral lysate were separated by SDS–PAGE on a 12% gel, followed by transferring proteins onto nitrocellulose membranes (Gelman Science). The membranes were probed with BST-2 antiserum (1:5000 dilution), anti-MuLV CA p30 antibody (1:5000 dilution), anti-Vpu serum (1:5000 dilution), or a mouse monoclonal antibody against actin (1:5000 dilution), followed by incubation with IRDye™ secondary antibodies (1:20,000). Protein bands were visualized with an Li-COR Odyssey instrument and quantified using the ImageJ automated digitizing program (NIH).

2.4. Immunofluorescence staining and confocal microscopy

For immunostaining, cells on coverslips were transfected as described above and then fixed in 4% paraformaldehyde. After two washes in 1× phosphate buffered saline, the cells were permeabilized with 0.5% Triton X-100. Staining was performed with primary antibodies including goat anti-MuLV CA p30 (1:1000 dilution) and BST-2 antiserum (1:1000 dilution). Anti-goat TRITC-conjugated antibody (1:500 dilution) and anti-rabbit FITC-conjugated antibody (1:500 dilution) were used as secondary antibodies. Images were recorded with a Leica TCS SPE, DM2500 confocal microscope (Leica Microsystems).

2.5. Protease digestion and PI-PLC or DTT treatment of cells and virions

To analyze the virus particles tethered to cell surface, HEK293T cells were cotransfected with proviral XMRV DNA and BST-2 expression plasmids. Free virions were harvested from the culture supernatants 48 h after transfection, as described above. The cells were washed twice with 1× phosphate buffered saline, once with subtilisin A buffer (10 mM Tris [pH 8.0], 1 mM $CaCl_2$, and 150 mM NaCl), and then treated with 1 mg/ml subtilisin A (Sigma) and either 100 mM DTT or 1 U/ml PI-PLC (Sigma) for 15 min at 37 °C. For trypsin treatment, cells were incubated with 3 mg/ml TPCK-treated trypsin or DMEM alone for 3 h at 37 °C. The proteases were quenched with DMEM containing 10% FCS, 5 mM PMSF, and 20 mM EGTA. The stripped supernatant was layered onto a 20% sucrose cushion and centrifuged, as described previously, to pellet virus particles.

3. Results

3.1. BST-2 significantly decreases XMRV release from mammalian cells

We first established Vero cell lines that stably express human BST-2. These cells, when transfected with XMRV DNA, produced significantly less viruses than the control cells (Fig. 1A). We then investigated whether XMRV is sensitive to inhibition by endogenous BST-2. HeLa cells, which express endogenous BST-2, were transduced with lentiviral vectors expressing control shRNA or BST-2 shRNA. As shown in Fig. 1C, the knockdown of endogenous BST-2 led to a significant increase in XMRV particle release.

BST-2 inhibits virus production by tethering nascent virus particles to the cell surface. Consistent with the antiviral mechanism, the results of imaging studies showed that BST-2 and XMRV Gag were colocalized at the plasma membrane in Vero cells stably expressing BST-2 (Fig. 1B). Knockdown of BST-2 also markedly decreased the number of HeLa cells that contained prominent intracellular accumulations of XMRV puncta that were localized at the cell surface and were also positive for BST-2 (Fig. 1D). We noted that knockdown of endogenous BST-2 in HeLa cells reduced XMRV Gag expression (Fig. 1C and D). This may result from the increased XMRV release that consumes more Gag proteins than in control HeLa cells. Together, these data demonstrate that both exogenous and endogenous BST-2 inhibit the release of XMRV virions.

3.2. Membrane binding and posttranslational modifications are essential forBST-2 to inhibit XMRV

BST-2 is a homodimeric, lipid raft-associated type II integral membrane glycoprotein. To determine how the structural domains and post-translational modifications affect the ability of BST-2 to inhibit XMRV, we generated BST-2 mutants, as depicted in Fig. 2A. These mutants either lacked the CT, TM, or GPI domains or had the glycosylation sites and the conserved cysteine residues mutated. We first investigated the subcellular localization of the wild-type and mutated BST-2 proteins. The results, shown in Fig. 2B, revealed that both the wild-type and BST-2 mutants were expressed at the plasma membrane and in intracellular locations. It was noted that the delCC deletion mutant was poorly expressed, as shown in the immunofluorescence and Western blot results (Figs. 2B and 3A).

The mutant delTM lacked the N-terminal transmembrane domain, but was still retained in the cell membrane by its GPI anchor. This mutant did not block viral particle release (Fig. 3A). Similarly, removal of the GPI anchor signal from BST-2 (delGPI) also abolished its antiviral activity (Fig. 3A). The delGPI mutant exhibited wild type-like glycosylation and subcellular distribution (Fig. 2B) [3]. Therefore, the N- and C-terminal membrane anchors of BST-2 are required for its anti-XMRV activity. Deletion of the coiled-coil

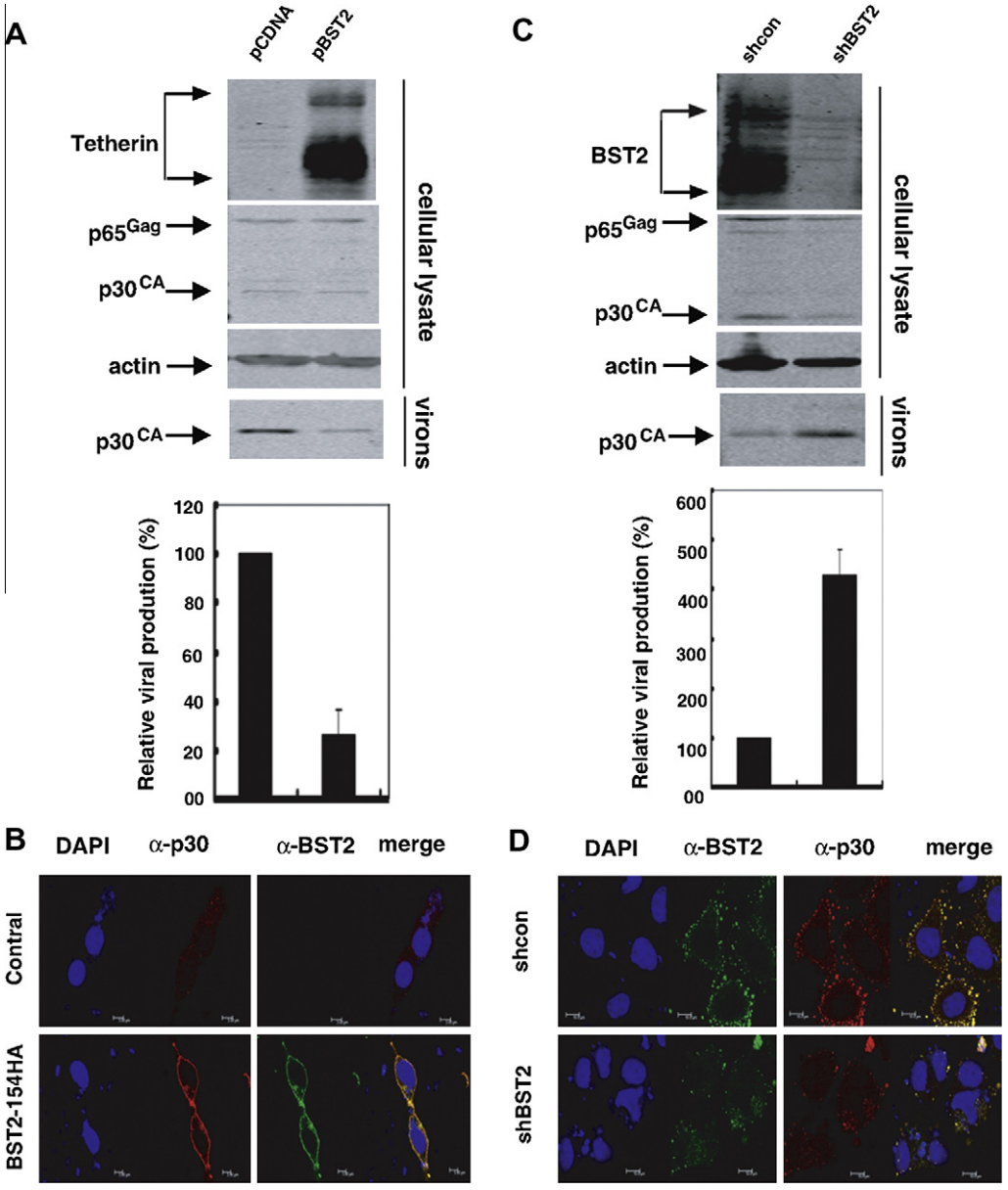

Fig. 1. Both exogenous and endogenous BST-2 restrict XMRV virion release. (A) Western blot analysis of the cellular lysates and the corresponding viral lysates following the transfection of XMRV proviral plasmid DNA into Vero cells that stably express human BST-2. The relative XMRV viral release was assessed by densitometric scanning of the specific bands using the ImageJ program (NIH). The level of XMRV virions released from control cells was set to 100%. The data shown are the averages of three independent experiments. (B) Immunofluorescence analysis (anti-CA p30, red; anti-BST-2, green) of Vero cells that stably express human BST-2 and are transfected with XMRV proviral DNA. The nuclei were stained with DAPI (blue). The images shown are from one representative experiment. The scale bar represents 5 μm. (C) Western blot analysis of the cellular lysates and the corresponding viral lysates following the transfection of XMRV proviral plasmid DNA into HeLa cells stably expressing shcon or shBST2. Relative XMRV viral release was assessed by densitometric scanning of the specific bands using the ImageJ program (NIH). The level of XMRV virions released from control cells was set to 100%. The data shown are the averages of three independent experiments. (D) Immunofluorescence analysis (anti-CA p30, red; anti-BST2, green) of HeLa cells cotransfected with plasmids expressing XMRV proviral DNA and either shcon or shBST2. The nuclei were stained with DAPI (blue). The images shown are from one representative experiment. The scale bar represents 10 μm.

domain (delCC) abrogated antiviral activity (Fig. 3B), suggesting that the coiled-coil domain is important for BST-2-mediated inhibition of viral release. It was noted that deletion of the cytoplasmic tail (delCT) did not affect the antiviral activity of BST-2 (Fig. 3A).

The extracellular domain of BST-2 has two putative N-linked glycosylation sites, N65 and N92, which are conserved in BST-2 proteins from humans, rhesus monkeys, rats, and mice [31]. The mutation of either N65 or N92 did not affect the anti-XMRV activity of BST-2. However, the N65/92A mutant could not inhibit XMRV release (Fig. 3A).

Human BST-2 possesses five cysteine residues: three in the extracellular domain and two in the intracellular domain. The three cysteine residues in the extracellular domain, namely C53, C63, and C91, are involved in BST-2 dimerization through the formation of disulfide bonds. To examine whether the dimerization of BST-2 is important for its anti-XMRV activity, we mutated the cysteine residues at positions 53, 63, and 91, either individually or in combinations, to generate the mutants C53A, C63A, C91A, C53/63A, C53/91A, and C63/91A. We also created a mutant, C53/63/91A, in which all three of these cysteine residues are replaced by alanine. As shown in Fig. 3B, BST-2 mutants with single and

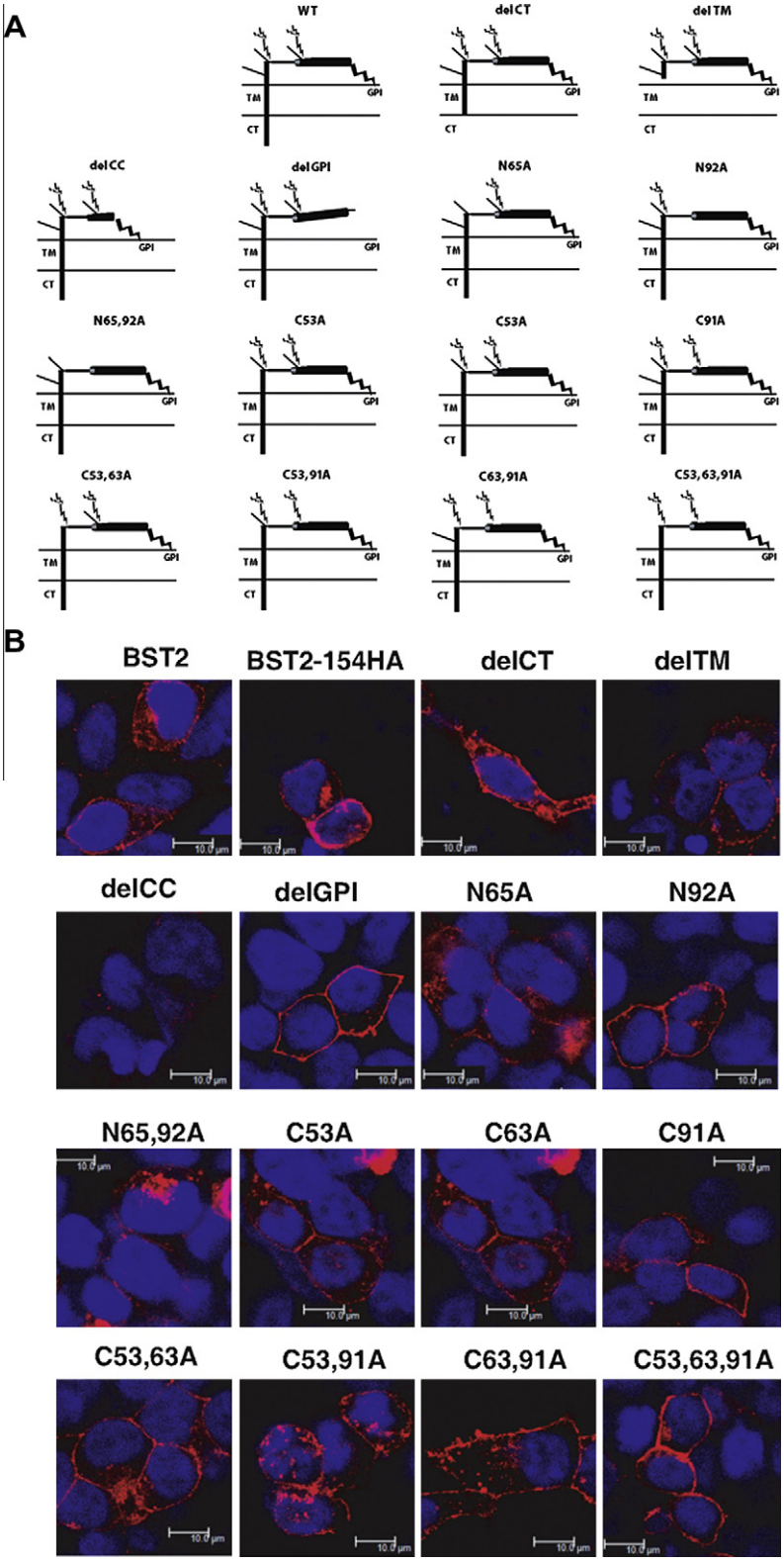

Fig. 2. BST-2 localization in HEK293T cells. (A) Schematic representation of the BST-2 mutants, including point mutations of the cysteine residues involved in ectodomain disulfide bond formation (C53, C63, and/or C91) or of the N-linked glycosylation sites (N65 and/or N92) and deletion mutations at the N-terminal cytoplasmic tail (delCT), ectodomain coiled-coil (delCC), or membrane anchors (delGPI and delTM). (B) Immunofluorescence staining was performed to detect the localization of BST-2 mutants (red, anti-HA) expressed in HEK293T cells. The nuclei were stained with DAPI (blue). The scale bar represents 5 μm. The images shown are from one representative experiment.

double substitutions at C53, C63, and C91 were still able to inhibit XMRV viral release from HEK293T cells. However, the mutation of all three cysteine residues (C53/63/91A) dramatically reduced BST-2 antiviral activity (Fig. 3B). These results demonstrate that the disulfide bond-mediated dimerization of BST-2 is important for its antiviral activity.

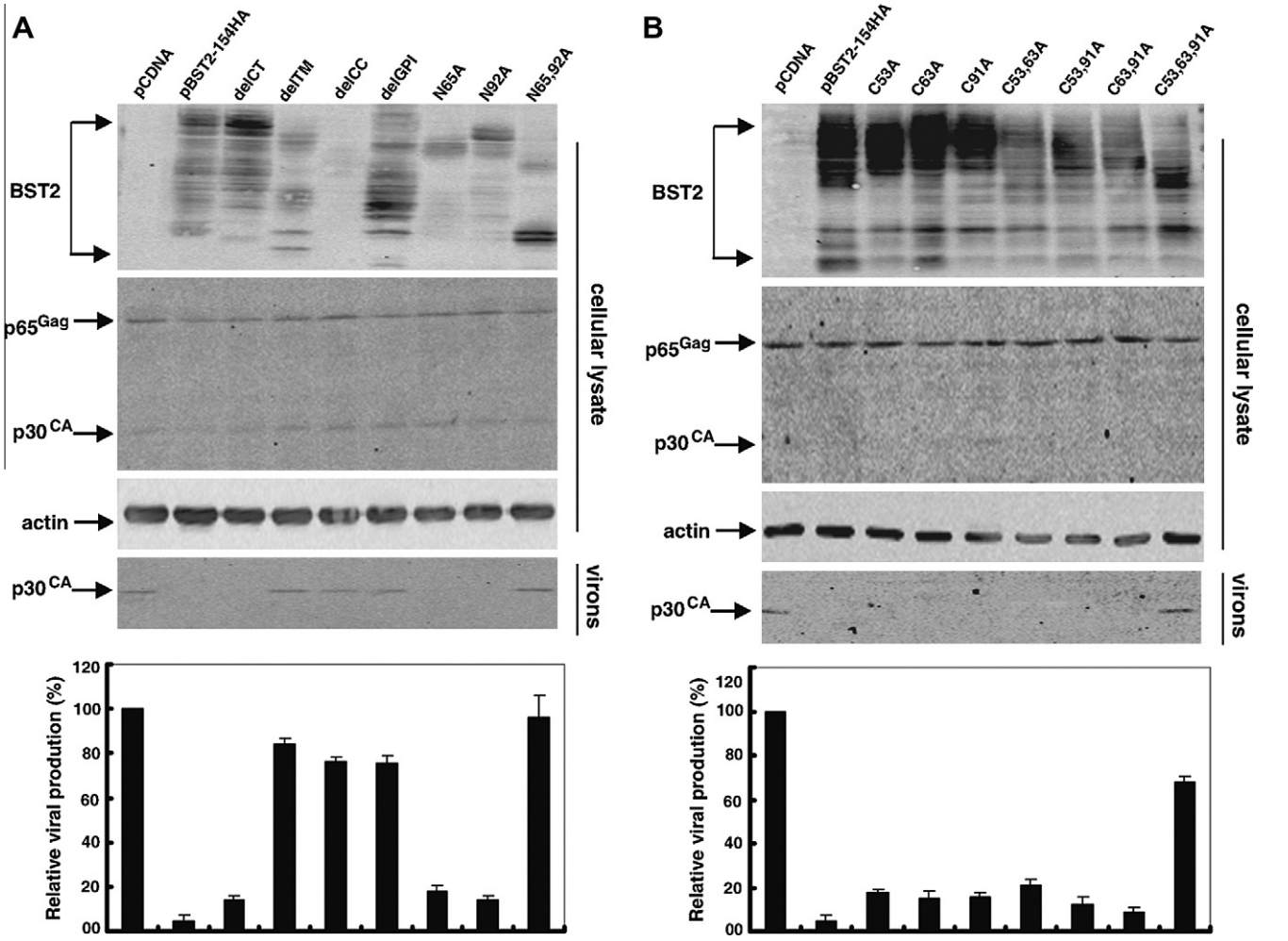

Fig. 3. Effects of wild-type and mutated BST-2 on XMRV release. Western blot analysis of the cellular lysates and the corresponding released viral lysates following the transfection of HEK293T cells with XMRV proviral DNA and plasmid DNA expressing wild-type BST2-154HA or various BST-2 mutants. Relative XMRV viral release was assessed by densitometric scanning of the specific bands using the ImageJ program (NIH). The level of XMRV virions released from cells cotransfected with the control vector was set to 100%. The data shown are the averages of three independent experiments. (A) Point mutations at the N-linked glycosylation sites (N65 and/or N92) and deletion mutations at the N-terminal cytoplasmic tail (delCT), ectodomain coiled-coil (delCC), or membrane anchors (delGPI and delTM) of BST-2. (B) Point mutations at the cysteine residues involved in ectodomain disulfide bonding (C53, C63, and/or C91) of BST-2.

3.3. Protease treatment releases BST-2-tethered XMRV particles from the cell surface

To determine whether BST-2 inhibits XMRV release and causes the accumulation of viral particles on the cell surface, we treated XMRV- and BST-2-expressing HEK293T cells with either subtilisin or TPCK-trypsin. As shown in Fig. 4, protease treatment effectively recovered the BST-2-retained XMRV virions. Interestingly, a trace amount of BST-2 protein was detected in XMRV viral particles as evidenced by their protection from protease digestion. This suggests that some BST-2 molecules is situated inside viral particles. These data are consistent with the proposed role of BST-2 in physically linking particles to the plasma membrane [31,32].

3.4. DTT and PI-PLC are unable to release the tethered virions

We next examined the importance of disulfide bond between BST-2 molecules in virion tethering. To this end, we incubated HEK293T cells expressing XMRV and BST-2 with 100 mM DTT for 15 min at 37 °C. The results showed that DTT treatment failed to release the tethered virions (Fig. 4). Next, we used phosphatidylinositol-specific phospholipase C (PI-PLC, 1 U/ml) to cleave the glycosylphosphatidyinositol (GPI) anchor. This treatment also failed to release the tethered virions (Fig. 4).

4. Discussion

In this study, we made several novel observations. First, through the results of our imaging studies, we confirmed that BST-2 tethers nascent XMRV particles to the cell surface. Second, we performed systematic mutagenesis studies to assess the contribution of BST-2 structural and modification features to virus inhibition. We concluded that the anti-XMRV activity of BST-2 depends on its unique membrane topology, cysteine-mediated dimerization and N-linked glycosylation. Third, we provided a detailed account of the subcellular distribution of all BST-2 mutants, illustrating the role of each BST-2 structural domain or modified residue in regulating BST-2 localization. Fourth, we observed that only subtilisin or trypsin, but not DTT or PI-PLC, is able to recover the tethered XMRV particles from the cell surface, suggesting that both the dimerization and double membrane anchor topology contribute to retaining XMRV particles at the cell surface.

The N-terminal transmembrane domain and the C-terminal GPI anchor at either end of the coiled-coil domain of BST-2 were re-

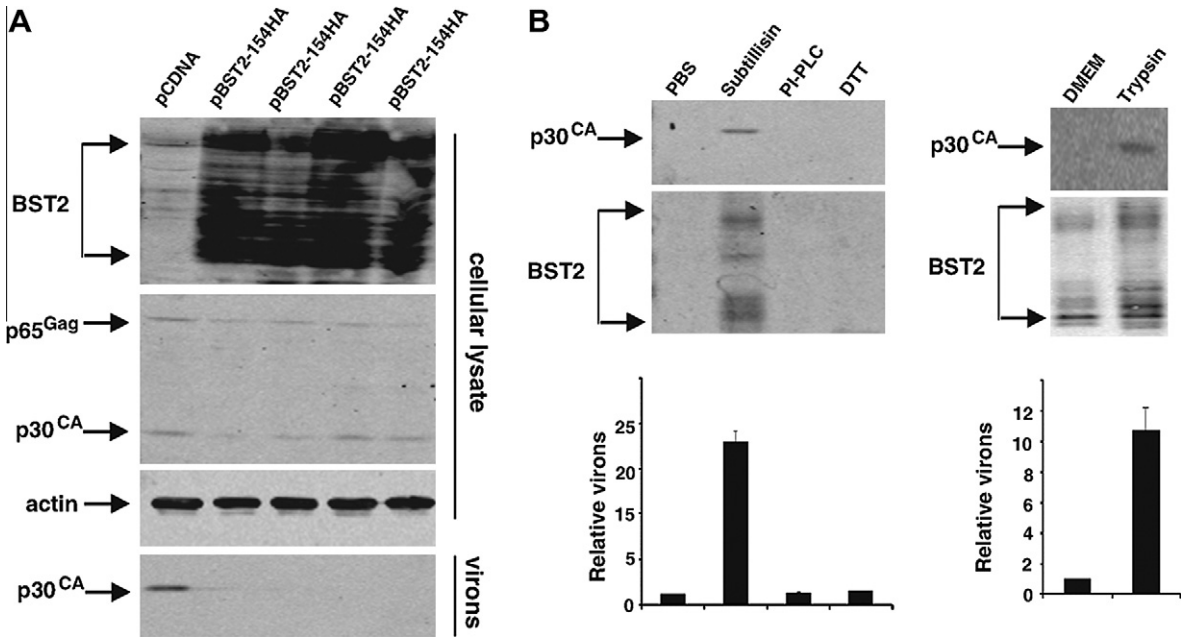

Fig. 4. Stripping the tethered virus particles from the cell surface. (A) Western blot analysis of the cellular lysates and the corresponding released viral lysates following the transfection of HEK293T cells with XMRV proviral DNA and plasmid DNA expressing BST2-154HA. (B) The transfected cells were washed twice with 1× phosphate buffered saline and then treated with 1 mg/ml subtilisin A (Sigma), 1 U/ml PI-PLC (Sigma) or 100 mM DTT for 15 min at 37 °C. For the trypsin treatment, 3 mg/ml of either TPCK-treated trypsin or DMEM alone was used for 3 h at 37 °C. The stripping treatment was quenched with DMEM containing 10% FCS, 5 mM PMSF, and 20 mM EGTA. The stripping supernatant was layered onto a 20% sucrose cushion and centrifuged as described in Section 2. The viral Gag protein and tetherin present in the pellets were assessed by Western blot. Relative amounts of XMRV virions stripped from the cell surface were assessed by densitometric scanning of the specific bands using the ImageJ program (NIH). The data shown are the average of three independent experiments.

ported to be essential for BST-2 antiviral function against HIV-1 [3,31]. Consistent with this, the delTM mutant, which lacks the N-terminal transmembrane domain and is retained in the cell membrane only by its GPI anchor, did not block XMRV release. Similar results were obtained with the delGPI mutant in which the GPI anchor signal was removed. Deletion of the coiled-coiled domain (delCC) eliminated the antiviral activity. Thus, the major structural features of BST-2, namely the N- and C-terminal membrane anchors and the coiled-coil domain, were required for inhibiting XMRV release.

Disulfide bonds between cysteines promote BST-2 dimerization. Dimerization of BST-2 is required for inhibiting HIV-1 release. [31,33]. but not for inhibiting the Lassa and Marburg viruses [34]. The mutant C53/63/91A that does not form cysteine-linked dimers lost their antiviral activity against XMRV. We further showed that the mutant N65/92A, which is not glycosylated, did not inhibit XMRV. These results suggest that the dimerization and glycosylation of BST-2 are important for its antiviral activity against XMRV.

We found that DTT and phosphatidylinositol-specific phospholipase C (PI-PLC) did not recover tethered XMRV from the cell surface. Similar attempts failed to release virus-like particles of Ebola virus that were retained on the cell surface by BST-2 [8]. One possibility is that DTT is not stable at 37 °C. Alternatively, the disulfide bonds are protected by the well-folded coiled-coil ectodoman, thus DTT is unable to reach the buried cysteines. In contrast to DTT, subtilisin and trypsin treatment released XMRV particles, which has also been reported for other viruses [32,35].

In conclusion, our work advances the understanding of BST-2 antiviral function. Elucidating the structural and biochemical basis of the antiviral function of BST-2 may ultimately lead to the development of antiviral therapeutic approaches against enveloped virus infection; such approaches may include enhancing BST-2 antiviral activity or disarming the viral antagonism against BST-2.

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