Positioning of Cysteine Residues within the N-terminal Portion of the BST-2/Tetherin Ectodomain Is Important for Functional Dimerization of BST-2*

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**Background:** BST-2 forms covalently linked dimers and inhibits virus release.

**Results:** Cysteine residues are not required for BST-2 homodimerization but are critical for antiviral activity.

**Conclusion:** Cysteine residues in the BST-2 ectodomain stabilize a structure capable of tethering virions to host cells.

**Significance:** Understanding the molecular basis of BST-2 virus-tethering activity will help us understand how cells try to minimize spreading infections.

BST-2/tetherin is a cellular host factor capable of restricting the release of a variety of enveloped viruses, including HIV-1. Structurally, BST-2 consists of an N-terminal cytoplasmic domain, a transmembrane domain, an ectodomain, and a C-terminal membrane anchor. The BST-2 ectodomain encodes three cysteine residues in its N-terminal half, each of which can contribute to the formation of cysteine-linked dimers. We previously reported that any one of the three cysteine residues is sufficient to produce functional BST-2 dimers. Here we investigated the importance of cysteine positioning on the ectodomain for functional dimerization of BST-2. Starting with a cysteine-free monomeric form of BST-2, individual cysteine residues were reintroduced at various locations throughout the ectodomain. The resulting BST-2 variants were tested for expression, dimerization, surface presentation, and inhibition of HIV-1 virus release. We found significant flexibility in the positioning of cysteine residues, although the propensity to form cysteine-linked dimers generally decreased with increasing distance from the N terminus. Interestingly, all BST-2 variants, including the one lacking all three ectodomain cysteines, retained the ability to form non-covalent dimers, and all of the BST-2 variants were efficiently expressed at the cell surface. Importantly, not all BST-2 variants capable of forming cysteine-linked dimers were functional, suggesting that cysteine-linked dimerization of BST-2 is necessary but not sufficient for inhibiting virus release. Our results expose new structural constraints governing the functional dimerization of BST-2, a property essential to its role as a restriction factor tethering viruses to the host cell.

BST-2/tetherin/CD317 is an interferon-induced protein capable of restricting the release of a variety of enveloped viruses, including lentiviruses such as HIV-1 (1, 2). It is part of a growing family of host restriction factors expressed by cells at least in part to fend off host pathogen infections. To counteract host restriction mechanisms, many viruses have evolved evasion strategies. These are often similar in principle but different on a mechanistic level. For instance, HIV-1, HIV-2, and simian immunodeficiency virus all antagonize BST-2 by down-regulating its cell surface expression. However, to neutralize BST-2 restriction, HIV-1 employs its Vpu protein, targeting the BST-2 TM domain, whereas HIV-2 uses its Env glycoprotein to target the BST-2 ectodomain. In addition, many simian immunodeficiency virus enzymes employ their Nef proteins to target the BST-2 cytoplasmic domain (for review, see Ref. 3). In the absence of these viral factors, BST-2 efficiently inhibits the release of cell-free virus particles by “tethering” them to the cell surface (3).

Tethering of otherwise mature virions to the plasma membrane of a virus-producing cell by BST-2 is accomplished by two membrane anchors that are separated by a largely α-helical coiled-coil ectodomain. One membrane anchor is formed by a transmembrane domain near the N terminus of the protein; the other membrane anchor is formed by a hydrophobic C-terminal domain (4). In rat BST-2, this C-terminal domain was previously reported to constitute a motif for GPI-anchor modification (4). However, no direct experimental data exist to demonstrate GPI anchor modification for human BST-2. Indeed, human BST-2 shares only ~33% amino acid identity with the rat protein. Although the overall structural features including the hydrophobic nature of the C terminus are conserved between human and rat proteins, recent evidence argues that the C terminus of human BST-2 may form a second transmembrane domain instead of being clipped off as part of a GPI anchor modification (5). Irrespective of the type of membrane anchor, BST-2 uses both anchors to tether viruses by inserting one end into the cellular plasma membrane, whereas the other

*The abbreviations used are: GPI, glycosylphosphatidylinositol; r.m.s.d., root mean square deviation.

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is present in the viral membrane with a slight preference for the C terminus to associate with the virion (6). Aside from its role as a viral tether, several recent papers have suggested that BST-2 may also have a role in antiviral cell signaling, activation of NFκB (7, 8), and antibody-dependent cell-mediated cytolysis (9, 10).

BST-2 is a 180-amino acid type II membrane protein expressed on lipid rafts at the cell surface and also in the trans-Golgi network (4, 11–14). BST-2 is modified by N-linked glycosylation at two positions in the ectodomain (Asn-65 and Asn-92) and, therefore, presents itself as a heterogeneous smear when separated by SDS-PAGE (4, 15–17). However, the importance of BST-2 glycosylation for its antiviral function remains to be firmly established (16, 17). BST-2 also contains three cysteine residues in the N-terminal region of its ectodomain, any one of which is sufficient for functional dimerization as shown by mutagenesis studies (16, 17). Structural studies of the BST-2 ectodomain have confirmed the importance of cysteine-linked dimerization for antiviral function (18–21). These models indicate that the disulfide bonds between parallel subunits that form dimers are important for stabilizing the structure of the irregular coiled-coil located in the C-terminal region of the ectodomain. Although these structural studies show BST-2-forming parallel dimers, they also suggest the formation of tetramers, the precise function and importance of which remain to be further determined (19–21). Extending these structural models, we recently showed that size and composition of the BST-2 ectodomain are highly flexible (22). We were able to reduce or increase its size as well as replace the BST-2-coiled-coil domain with a coiled-coil region from a heterologous protein (vimentin), all while maintaining antiviral function. Interestingly, the success of these modifications appeared to be dependent on the conservation of a coiled-coil structure that maintained the dimer interface between the parallel molecules.

In an extension of these studies, we investigated here the importance of cysteine positioning on the ectodomain for functional dimerization of BST-2. Starting with an inactive, cysteine-free monomeric form of BST-2, individual cysteine residues were reintroduced throughout the ectodomain at positions predicted to form or to not form disulfides based on the available x-ray crystal structures (18–21). The resulting BST-2 variants were tested for expression, dimerization, surface presentation, and inhibition of HIV-1 virus release. Our results demonstrate significant flexibility in the positioning of cysteine residues with regard to functional BST-2 dimerization even though the propensity to catalyze dimerization generally decreased with increasing proximity of the cysteines to the C terminus of the BST-2 ectodomain. Importantly, our data indicate that BST-2 dimerization is not sufficient for inhibition of virus release as not all dimerization-competent BST-2 variants were functional in our virus release assay. Our results expose new structural constraints governing the functional dimerization of BST-2, a property essential to its role as a restriction factor tethering viruses to the host cell.

EXPERIMENTAL PROCEDURES

Plasmids—Constructs for the expression of wild type BST-2 (pcDNA-BST-2) and a BST-2 variant, in which cysteines Cys-53, Cys-63, and Cys-91 were mutated to alanine (pcDNA-BST-2 C3A), have been described previously (16). For the generation of individual cysteine substitutions, pcDNA-BST-2 C3A was used as a template for QuikChange mutagenesis (Stratagene, La Jolla, CA). All constructs were verified by sequence analysis. The full-length HIV-1 molecular clone pNL4–3 and the Vpu deletion mutant pNL4–3/Udel have been described previously (23, 24). The plasmid pEGFP-N1 was used for GFP expression (Clontech, Mountain View, CA).

Antisera—Rabbit polyclonal BST-2 antiserum, directed against the extracellular portion of BST-2, has been described previously (16, 25). HIV-1 Gag was detected using HIV-IG from pooled human serum (National Institutes of Health AIDS Reagent Program; catalog #3957).

Cell Culture and Transfections—293T cells were propagated in Dulbecco’s modified Eagles medium (DMEM) containing 10% fetal bovine serum. For transfections, 293T cells were grown in 25-cm² flasks to ~80% confluency (~3 × 10⁶ cells). Cells were transfected using Lipofectamine PLUS™ (Invitrogen) following the manufacturer’s recommendations. A total of 5 μg of plasmid DNA per 25 cm² flask was used. Where appropriate, empty vector DNA was used to adjust total DNA amounts. TZM-bl cells are derived from HeLa cells and contain the CXCR4 coreceptor and a luciferase indicator gene under the control of the HIV-1 long terminal repeat (LTR). TZM-bl cells were obtained from John Kappes through the National Institutes of Health AIDS Reagent Program (catalog #8129).

Immunoblotting—For immunoblot analysis of BST-2 under reducing or non-reducing conditions, cell extracts were prepared as follows: cells were scraped, washed once with PBS, and transferred to 1.5-ml reaction tubes. Cell pellets were suspended in 300 μl of CHAPS/DOC buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 100 mM NaCl, 0.5% CHAPS, 0.2% deoxycholate (both from Sigma)) and incubated for 5 min on ice after brief vortexing. Samples were then warmed to 37 °C for 20 s to complete cell lysis, and insoluble material was removed by centrifugation in a minicentrifuge (3 min, 16,000 relative centrifugal force). Supernatants were transferred to fresh reaction tubes (2 tubes with 50% lysis each) and mixed with an equal volume of reducing sample buffer (4% sodium dodecyl sulfate, 125 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue) or non-reducing sample buffer (same as above except without 2-mercaptoethanol). Samples were heated for 5 min at 95 °C with occasional vortexing and subjected to SDS-PAGE. Proteins were transferred to PVDF membranes and reacted with appropriate antibodies as described in the legend for Fig. 2. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare), and proteins were visualized by enhanced chemiluminescence (Clarity™ Western ECL; Bio-Rad). Images were acquired on a ChemiDoc™ MP Imaging System (Bio-Rad).

Formaldehyde Cross-linking of BST-2—To stabilize non-covalent oligomers of BST-2 for subsequent SDS gel analysis and immunoblotting, transfected cells were harvested by scraping and washed once with PBS. Cells were then pelleted (5 min, 500 relative centrifugal force), transferred to a 1.5-ml reaction tube, and suspended in 1 ml of serum-free DMEM containing 1% formaldehyde (diluted from a 37% stock). Samples were incubated at 37 °C for 20 min after which the reaction was stopped.
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by the addition of 100 μl of 1.25 M glycine in PBS. Cells were then pelleted, lysed in CHAPS/DOC buffer as described above, and combined with an equal volume of reducing sample buffer. Samples were heated for 5 min at 65 °C before SDS-PAGE.

BST-2 Surface Expression and FACS Analysis—Determination of BST-2 surface localization was performed as described (16). Briefly, 293T cells were transfected with 0.1 μg of BST-2 plasmid together with 1 μg of pEGFP-N1 as a transfection control. Twenty-four hours later, cells were washed once with ice-cold PBS, harvested in ice-cold 20 mm EDTA-PBS, and then washed twice with 1% BSA-PBS. Cells were then treated for 10 min with 50 μg of mouse IgG (Millipore, Temecula, CA) to block nonspecific binding sites. Cells were then incubated with primary antibody against BST-2 for 30 min at 4 °C. After 2 washes with 1% BSA-PBS, cells were incubated with allophtocyanin-conjugated anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove PA) for 30 min at 4 °C in the dark. Cells were then washed twice with 1% BSA-PBS and fixed with 1% formaldehyde in PBS. Cells were analyzed on FACS Calibur (BD Biosciences), and data analysis was performed using FlowJo (Tree Star, San Carlos CA) and gating of GFP-expressing cells.

Viral Infectivity Assay—293T cells were transfected with the indicated amounts of BST-2 constructs together with 4.5 μg of pNL4–3/Udel. Virus-containing supernatants were harvested 24 h after transfection and clarified by centrifugation (3 min at 1500 × g) and filtration (0.45 μm) to remove cellular debris. Equal volumes of viral supernatants were then used to infect 5 × 10⁴ TZM-bl cells in a 24-well plate. After 48 h, media was removed, and cells were lysed in 300 μl of 1× Reporter Lysis Buffer (Promega Corp., Madison WI) and subjected to freeze-thaw cycle at −80 °C. Luciferase activity in the lysates was determined by combining 5 μl of lysate with 20 μl of Steady-Glo luciferase substrate (Promega) and by measuring light emission using a Modulus ii Microplate reader (Turner Biosystems Inc., Sunnyvale CA).

Structural Modeling—The human BST-2 structure (Protein Data Bank [PDB] code 3MQB) was used to create models of the cysteine substituted BST-2 mutations within YASARA 12.7.16 (26). The BST-2 dimer was truncated to 10 amino acids on either side of the target amino acid. Where appropriate a disulfide bond was introduced between the cysteines in each subunit of the BST-2 dimer. Each model was run at a temperature of 310 K, 0.9% NaCl, pH 7.4, using an AMBER03 force field until the change in backbone root mean square deviation with time was essentially zero (27). For each mutant run within YASARA, the “wild type” model was run as a control where the sequence was unchanged and no disulfide bond was present. The r.m.s.d. values for all atoms and the Ca atoms were calculated within YASARA.

Crystal Structure Analysis—B-factors were determined from data deposited in the Research Collaboratory for Structural Bioinformatics (28).

RESULTS

Role of Cysteine Positioning in BST-2 Dimerization—Cysteine-linked dimerization of BST-2 is essential for its antiviral activity, and this requires the presence of at least one of three cysteine residues (Cys-52, Cys-63, Cys-91) located in the N-terminal half of the BST-2 ectodomain (16, 17). This is also true for a partially active “artificial” BST-2 in which dimerization was achieved by the presence of two cysteine residues that are part of an ~30 residue fragment of the ectodomain of the transferrin receptor, which, like BST-2, forms cysteine-linked dimers (17, 29).

However, the crystal structures available for the ectodomain of BST-2 show some structural flexibility, which is consistent with recent functional studies on the size and conservation of the BST-2 ectodomain (18–22). In our previous work we found that residues 50–100 in the BST-2 ectodomain are flexible, whereas residues 101–150 form a rigid coiled-coil structure (22). The coiled-coil region of BST-2 encompasses several heptad repeats (Fig. 1) in which positions a, d, and e of the heptads are typically small hydrophobic residues that pack the coiled-coil together in a knobs into holes manner. On the basis of these structural insights we wondered how critical the positioning of the cysteine residues on the ectodomain was for the assembly of functional BST-2 dimers. We performed a mutagenesis study where cysteine residues were reintroduced at multiple positions along the ectodomain of a BST-2 variant, C3A, in which all three naturally occurring cysteines required for dimerization had previously been mutated to alanine (16). Based on the crystal structures, we hypothesized that positions where the side
chain faces the other subunit of the dimer (e.g. a and d positions in the heptads as indicated in Fig. 1) would tolerate a cysteine substitution and form a stable, functional dimer. However, introduction of a cysteine at a position facing away from the other subunit would have a low probability of disulfide bond formation and/or result in a non-functional dimer. Therefore, we chose amino acid positions that we predicted to be amenable to the formation of functional disulfide bridges and an adjacent position that would not be expected to form functional cysteine-linked dimers. This selection of amino acids mutated in this study is shown in Fig. 1 (identified as raised blue letters). These include several residues occupying positions a and d in the heptad repeats of the coiled-coil region, positions that would be favorable for interactions between the subunits of the dimer.

To test the ability of individual single cysteine variants to dimerize, we employed two assays. In the first assay, we tested the cysteine replacement mutants for differences in electrophoretic mobility under reducing (Fig. 2A) or denaturing but non-reducing conditions (Fig. 2B). For this assay, expression vectors were transfected into 293T cells, which do not express endogenous BST-2. Proteins were extracted and analyzed under either reducing or non-reducing conditions by heating to 95 °C in sample buffer either containing or lacking the reducing agent 2-mercaptoethanol (Fig. 2, A and B). Heating samples in the presence of 2-mercaptoethanol will break up both non-covalent and cysteine-linked covalent interactions, whereas in the absence of 2-mercaptoethanol only non-covalent interactions will be dissolved, whereas cysteine-linked dimers will remain intact.

In the second assay we tested the same mutants for their ability to form non-covalent dimers using formaldehyde cross-linking for stabilization (Fig. 2C). In this assay cells were treated with formaldehyde (1%) for 20 min at 37 °C before cell lysis and heating to 65 °C in reducing sample buffer (Fig. 2C). This assay allows for the identification of protein-protein interactions but does not discriminate between non-covalent or covalent interactions.

Fig. 2, A–C, shows a representative selection of mutants. The combined results for all mutants tested in our study are tabulated in Fig. 2D. As previously observed, wild type BST-2 when analyzed under reducing conditions migrates as a smear of heterogeneously glycosylated forms with apparent molecular weights ranging from 28 to 40 kDa (Fig. 2A, lane 2; green box). However, under non-reducing conditions, wild type BST-2 migrates at 55–70 kDa (Fig. 2B, lane 2; red box), indicating efficient dimer formation. As reported previously, the cysteine-deficient C3A variant was unable to form disulfide-linked dimers and remained in the monomeric state (Fig. 2B, lane 3; green box). Although dimerization of wild type BST-2 was virtually quantitative, most of the cysteine scanning mutants had intermediate phenotypes with varying ratios of monomer to cysteine-linked dimer conformations. For instance in the case of Q78C (lane 5) and N141C (lane 15), at least 50% of the protein formed disulfide-linked dimer. Other variants such as Q110C (lane 9), G118C (lane 11), or E140C (lane 14) appeared to be largely unable to form disulfide-linked dimers and exhibited electrophoretic mobilities similar to C3A. Interestingly, when proteins were analyzed for their ability to oligomerize using formaldehyde cross-linking, we found that virtually all mutants, including the C3A triple cysteine mutant, were at least partially able to form homo-oligomers (Fig. 2C). Some mutants, e.g. Q110C or V113C (Fig. 2C, lanes 9 and 10), were less effective in dimerization than the cysteine-deficient C3A variant, suggesting that in some instances the introduction of a cysteine residue interfered with BST-2 oligomerization. As expected, most residues located at positions a or d of the coiled-coil heptad repeats (see Fig. 1) were able to form at least partial cysteine-linked dimers. Of note, even under reducing conditions (Fig. 2A), BST-2 C3A and many of the single cysteine variants exhibited electrophoretic mobility distinct from that of wild type BST-2. This change in mobility is due to differential glycosylation at one of the two glycosylation sites, as discussed further below.

**Cell Surface Expression of BST-2 Cysteine Variants**—To restrict HIV-1 viral release, BST-2 must be transported to the cell surface to sites of viral budding. To assess the effect of changes in cysteine positioning on intracellular trafficking and, in particular, on cell surface expression, we employed flow cytometry for surface detection of transiently expressed BST-2 variants. As seen in Fig. 3A, wild type BST-2 (wt) was readily detected on the surface of non-permeabilized 293T cells (red trace in all panels). Similarly, the cysteine-deficient C3A variant was efficiently expressed at the cell surface (green trace in panel a). BST-2 C3A exhibited consistently higher cell surface levels relative to BST-2 wt. Importantly, the vast majority of our cysteine scanning variants were expressed at the cell surface at levels equal to or higher than the wild type protein. Only a representative selection of BST-2 variants is shown in Fig. 3A. The results for all tested variants are tabulated in Fig. 3B. The phenomenon of increased surface expression of BST-2 mutants is not without precedent as we had previously noted a similar effect resulting from mutation of the natural cysteines in the BST-2 ectodomain (16). Up-regulation of BST-2 surface expression was also reported for other non-cysteine ectodomain mutants (30). The reason for the more efficient surface presentation of most BST-2 variants relative to wild type protein is not clear; however, it is not explained by differences in intracellular protein expression (not shown). Therefore, together with the results from Fig. 2, our data indicate that efficient cell surface presentation does not depend on efficient cysteine-linked BST-2 dimerization.

**Functional Analysis of Cysteine Variants**—To assess the ability of our BST-2 cysteine variants to inhibit virus release, 293T cells were transfected with increasing amounts of BST-2 vectors in the presence of constant amounts of the Vpu-deficient pNL4–3/Udel proviral DNA. Viral supernatants were collected 24 h after transfection and used to infect TZM-bl indicator cells. Relative viral titers were determined by measuring the virus-induced luciferase expression from duplicate infections. As expected, release of Vpu-deficient HIV-1 virions decreased in a dose-dependent manner in the presence of increasing amounts of BST-2 wt (Fig. 4A, solid circles in all panels), whereas the dimerization-incompetent BST-2 C3A variant was unable to inhibit viral release (Fig. 4A, panel a; open circles). All of the cysteine scanning variants were tested in this manner.
Data for a representative selection are shown in Fig. 4A, and the results for the complete set are summarized in Fig. 4B. Some BST-2 variants were able to restrict virus release with similar efficiency as BST-2 wt (e.g. Q72C, A77C, F81C), whereas others exhibited partial activity (e.g. V84C, G109C), and a significant portion of the tested variants was unable to restrict virus release at all (e.g. V113C, L137C, V143C).

The results of both dimerization (Fig. 2) and restriction assay (Fig. 4) are schematically summarized in Fig. 5. In this figure the height of the bar reflects the efficiency of virus release, whereas the colors indicate the propensity of the respective BST-2 variant to form cysteine-linked dimers as indicated in the figure. The figure shows that complete cysteine-based dimerization was not required for partial (e.g. M96C, G109C) or even full (F81C) restriction capacity of BST-2 (Fig. 5, green bars). Nevertheless, variants displaying the highest antiviral activity generally also exhibited efficient cysteine-linked dimerization (blue bars). Not surprisingly, we did not find any dimerization-in-
competing BST-2 variants that retained antiviral activity (Fig. 5, red bars). Dimerization itself, however, was not sufficient for antiviral activity. For example, Q78C showed significant dimerization (Fig. 2, B and C, lane 5) was efficiently expressed at the cell surface (Fig. 3A) but completely failed to restrict virus release (Fig. 4A). Generation of functional dimers appeared to be restricted to cysteines near the N terminus, with the most active variants containing cysteines N-terminal to residue 85. In fact, we only identified two positions C-terminal to residue 100 in BST-2 (G109C, N141C) where reintroduction of a cysteine restored partial antiviral activity. This suggests that the N-terminal region of the BST-2 ectodomain may indeed be more flexible and more accommodating to positional changes of the disulfide bonds, whereas the C-terminal half may be rigid and less able to accommodate cysteine bonds without compromising structural features required for antiviral function.

**Molecular Modeling of the Effects of Cysteine Insertions**—To obtain structural insights into the effects of cysteine insertions along the BST-2 ectodomain, molecular modeling was performed on a representative set of the variants analyzed above. Fig. 6 shows the predicted structures of three sets of BST-2 variants with cysteines introduced at adjacent positions. For each variant, a region of 10 amino acids on either side of the respective cysteine was determined using YASARA (26). This length was chosen to ensure there was at least one complete heptad on either side of the target amino acid. The structure of the wild type protein without cysteine replacement is shown in red in all panels whereas the structural model of the same fragment, now with a cysteine residue in the center, is shown in blue. The two structures in each panel represent the two helices of the BST-2 dimer. The V84C and E85C pair (Fig. 6, panels I and II) both revealed efficient cysteine-based dimerization in our experimental analysis (Fig. 2B). However, only the V84C variant was functional, whereas the E85C variant was defective (Fig. 4A). In the case of V84C where functional dimerization was accomplished, the insertion of cysteines did not predict any obvious changes in our structural model (Fig. 6, panel I). This is supported by the small changes in the backbone compared with the non-substituted model (r.m.s.d. Cα atoms 0.6 Å). In contrast, insertion of a cysteine at the adjacent position, E85C (panel II), predicted a significant structural change (r.m.s.d. Cα 4.8 Å), which might explain the loss of the antiviral property. Likewise, in the G109C variant, which formed partial dimers, the cysteine/disulfide bond insertion was well tolerated with
little change in the predicted overall structure (r.m.s.d. Cα 0.8 Å) (Fig. 6, panel III). Nevertheless, this variant was not quite as active as the wild type protein as evidenced by the fact that comparable restriction required transfection of more G109C DNA (Fig. 4 Ad). Introducing a cysteine residue at the adjacent position (Q110C) turned out to be unfavorable for the formation of cysteine-linked dimers as it would interfere with the formation of a stable coiled-coil (r.m.s.d. Cα 3.2 Å) (Fig. 6, panel IV). The same is true for the introduction of a cysteine at position Glu-140, where the formation of a cysteine-bridge was predicted to induce significant structural changes (Fig. 6, panel V) explaining why the full-length protein remained largely monomeric and was non-restrictive (r.m.s.d. Cα 2.9 Å). In contrast, introduction of a cysteine at the adjacent Asn-141 was not predicted to cause significant structural changes (Fig. 6, panel VI), offering a possible explanation as to why this variant formed partial cysteine-linked dimers in our experimental analysis (r.m.s.d. Cα 1.5 Å). Indeed, the BST-2 N141C reproducibly exhibited partial antiviral activity that in some experiments reached up to 50% of the wild type activity. Overall, however, introducing cysteine residues within the C-terminal half of the BST-2 ectodomain was generally not very effective for the restoration of antiviral activity.

**DISCUSSION**

Following the identification of BST-2 as an inhibitor of virus release that is targeted by HIV-1 Vpu (1, 2), expression of BST-2 has been associated with the inhibition of the release of a variety of enveloped DNA and RNA viruses. A current working model proposes that inhibition of virus release is accomplished through affixing BST-2 to the cellular and viral membranes via its two membrane anchors with the coiled-coil BST-2 ectodomain functioning as a tether to bridge the gap between the two membranes (for review, see Refs. 31 and 32). In support of such a relatively simple mechanical function of BST-2, artificial tetherin with structural features similar to those of authentic BST-2 (i.e. two membrane anchors with an intervening coiled-coil domain and the ability to form cysteine-linked dimers) but virtually no primary amino acid sequence identity was shown to be capable of trapping nascent viral particles at the cell surface just like BST-2 albeit with much reduced efficiency (17). Additionally, the relative tolerance of BST-2 to changes in the size or primary sequence of its ectodomain is consistent with a simple mechanical function of BST-2 (5, 22, 30).

On the other hand, the fact that replacing or supplementing the rigid coiled-coil ectodomain by less rigid non-coiled-coil sequences destroys the tethering function of BST-2 (22) and that the formation of cysteine-linked dimers is a structural requirement for the inhibition of HIV-1 particle release (16, 17) demonstrates that there are limits to the flexibility of the ectodomain. The actual number and the positioning of cysteines on the BST-2 ectodomain as demonstrated in the current study are relatively flexible. In fact, some viruses such as Lassa, Mar-

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**FIGURE 4. Functional analysis of cysteine scanning mutants.** A, 293T cells were transfected with constant amounts of pNL4–3/Udel (4.5 μg) and increasing amounts of BST-2 variants (0–0.5 μg). Viral supernatants were collected 24 h after transfection and used for the infection of TZM-bl indicator cells. Virus-induced luciferase activity was measured 48 h later. Luciferase activity obtained in the absence of BST-2 (0 μg) was defined as 100%. B, the full panel of BST-2 cysteine variants indicated was investigated as in panel A, and the results of two to five independent experiments are summarized. The ability of these variants to restrict is expressed relative to WT (+++) and C3A (−). Lowercase letters identify the corresponding histogram in panel A.
burg, or Foamy viruses do not even seem to require cysteine-linked dimerization of BST-2 (33, 34). The reason why inhibition of HIV-1 requires the formation of cysteine-linked dimers, whereas other viruses can do without, remains unclear. One possible explanation may be found in our observation that all BST-2 variants, irrespective of their ability to form functional (or non-functional) cysteine-linked dimers, including the cysteine-deficient C3A variant, have the ability to assemble into non-covalent dimers or oligomers as documented by formaldehyde cross-linking. Although it is conceivable that the formation of covalent dimers affects the subcellular localization of BST-2, it is unlikely that this would play a role here as all cysteine variants engaged in the current study were efficiently expressed at the cell surface. Furthermore, given the overall reducing environment inside cells, it is likely that cysteine-linked dimers do not form until after the protein reaches the surface and is exposed to the oxidizing extracellular environment. Thus, it is more likely that the cysteine bridges provide structural support necessary for the tethering of HIV-1 virions to the plasma membrane. The fact that wild type BST-2 encodes three cysteines in its ectodomain, yet any one of them alone is sufficient for the formation of functionally active BST-2 (16, 17) rules out the possibility that BST-2 forms higher order covalently linked membrane complexes as a prerequisite for tethering activity. Nevertheless, our data support the notion that aside from the formation of covalent dimers, the overall quaternary structure of BST-2 oligomers is critical for its antiviral property.

Our finding that not all BST-2 variants capable of forming cysteine-linked dimers are capable of inhibiting HIV-1 release can be explained by a need for maintaining proper alignment of the parallel coiled-coils. As shown in Fig. 6, placing cysteines into positions that allowed cross-linking of BST-2 without a need for a conformational strain on the helix were more likely to assemble into functional BST-2 dimers than constructs encoding cysteines in positions where the side chains of the residues point away from each other on the coiled-coil. Of note, even though placing cysteine residues in certain positions in the C-terminal half of the ectodomain supported at least some level of dimer formation, the likelihood of a variant to form cysteine-linked dimers decreased the farther from the N terminus it was located. This is consistent with the previously noted overall greater flexibility of the N-terminal portion of the ectodomain (22) but, again, points to a more specific function of the ectodomain above and beyond a mere mechanical tether.

Several groups have published x-ray crystal structures of the BST-2 ectodomain (18–21) producing a variety of different structural models (Fig. 7). Analysis of the crystal packing of structures 3NI0, 2X7A, 3MQB, and 3MQC showed that in each case the N-terminal portion of BST-2 packed against an adjacent BST-2 dimer in the asymmetric unit or in the case of 3MQB a symmetry-related molecule. This packing restricts the conformations of the N terminus of the dimer, a requisite for obtaining electron density in this region of the protein from crystals. When this packing was absent, the B factors were noticeably higher, which reflects the thermal motion of the

FIGURE 5. Summary of the cysteine scanning analysis. Shown is the amino acid sequence of the BST-2 ectodomain. Cysteines present in wild type BST-2 are indicated in red. Bars represent positions where cysteine residues were inserted. The color of the bars indicates the dimerization potential as judged from the analysis of non-reducing protein gels (Fig. 2B). The height of each bar reflects the efficiency of virus release in the presence of a given BST-2 variant as judged from the dose-response analysis (Fig. 4). Virus release in the absence of BST-2 is defined as 100%. TM1 denotes the location of the N-terminal transmembrane domain. TM2/gpi denotes the beginning and direction of the C-terminal hydrophobic domain, which may act as a second TM domain or constitute a signal for GPI-anchor modification. N1 and N2 indicate the sites for N-linked glycosylation. Asterisks above the bars indicate variants for which experimental data are shown in the previous figures.
amino acid within the crystal and can be interpreted to indicate regions of higher flexibility or of low occupancy protein conformations, which were not modeled during the analysis of the diffraction data. In both scenarios, the structure of BST-2 is not in a static conformation. These data suggest that there are one or more areas within BST-2 that act like a hinge allowing the position of the N terminus to move relative to the position of the C terminus (21, 22). Alignment of the crystal structures indeed supports a hinge point around amino acid 80 as noted previously (22). Given that BST-2 may bridge the viral particle and the host cell membrane, it is conceivable that the flexibility of the N terminus plays a role in early viral tethering by allowing BST-2 to accommodate changes in the curvature of the cell membrane during viral budding.

The specific role of the disulfide between the BST-2 monomers is still not clear. The fully reduced BST-2 ectodomain is known to be less stable structurally than the (at least partially) oxidized form (18, 21); however, whether this is due to changes in the orientation of the monomers that form the coiled-coil or affinity of the two monomers for each other is not clear. Introduction of a disulfide bond in the coiled-coil may alter either orientation or affinity resulting in decreased viral tethering activity of BST-2.

One final point concerns the glycosylation of BST-2. As can be seen in Fig. 2, some BST-2 variants displayed changes in their electrophoretic mobility relative to the wild type protein. For instance, BST-2 C3A, Q78C, or G109C among others exhibited reduced mobility on SDS-PAGE with apparent molecular

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**Relevance of Cysteine-linked Dimerization of BST-2**

**FIGURE 6. Molecular modeling of the effects of cysteine insertions.** The structure of BST-2 (PDB 3MQC (19) was truncated to 10 amino acids on either side of the site of cysteine replacement in YASARA. The target residue was substituted with cysteine, and a disulfide bond was introduced between the thiols (blue). The corresponding wild type sequence is shown in red. The model was solvated in 0.9% NaCl at pH 7.0 and warmed to 37 °C. The model was then allowed to relax using an AMBER03 force field until the r.m.s.d. was stable with respect to time. For the control (red), we did not introduce a cysteine or a disulfide into the model; however, the simulation was run under identical conditions. Three pairs of BST-2 variants are shown with their respective Ca r.m.s.d. and the r.m.s.d. for all matched atoms between the mutant and the wild type models. The functional properties of the BST-2 variants are discussed in “Results.” With the exception of panel IV, all structures containing cysteines (blue) are shown in their disulfide-bridged conformation. The cysteine-containing structure in panel IV is shown as a non-disulfide-linked dimer (see “Results”).
masses of 35–45 kDa. Mutation of the two glycosylation sites in BST-2 C3A, either individually or in combination, revealed that mutation of glycosylation site N1 (i.e. Asn-65) eliminated the difference in electrophoretic mobility (data not shown). This suggests that the change in mobility is caused by differential modification of the carbohydrates at the N1 site and could reflect changes in the trafficking pathway of the proteins or could indicate other structural changes in the protein affecting its migration profile (16). Interestingly, for some variants such as A77C and V84C, insertion of a cysteine residue restored wild type electrophoretic mobility. The reason for the position-sensitive impact of cysteine residues on the glycosylation profile of

FIGURE 7. Analysis of BST-2 x-ray crystal structures. Several x-ray crystal structures of BST-2 were pulled from the PDB to compare asymmetric units as well as B-factors. The PDB ID for these x-ray crystal structures are 3NWH, 3N10, 2X7A, 2X7G, 3MQB, 3MQC, and 3MQ7. For each crystal structure the B-factor was plotted against residue number. The N and C termini are labeled. We found that structures 3NWH, 3MQC, 3MQ7, and 2X7A all have another protein in the asymmetric unit that interacts in the N terminus. The structures 3N10, 2X7G, and 3MQB do not interact this way. Interestingly, comparison of the B-factor plots shows that this latter group with the exception of 3MQB have higher B-factors, indicating more flexibility, at the N terminus. 3MQB interacts with symmetry-related BST-2 molecules in a similar fashion to 3NWH, 3MQC, 3MQ7, and 2X7A. This is the likely cause of the low B-factors in the N terminus for this structure. These data support the suggestion that the N terminus of BST-2 is flexible, and this flexibility is reduced by interactions between BST-2 protein dimers in the crystal.
BST-2 is not clear. However, the altered mobility did not correlate with the ability to form cysteine-linked dimers (cf. Fig. 2B, Q78C, G109C, L137C, or N141C) or the surface presentation of the proteins. However, all variants with strong antiviral properties (e.g. A77C, V84C) exhibited electrophoretic mobility similar to wild type BST-2. We previously reported that glycosylation of BST-2 is not a prerequisite for its antiviral activity (16). In addition, we found that BST-2 C3A, like all other cysteine variants, was transported to the cell surface at least as efficiently as wild type BST-2. Although the functional significance of this phenomenon remains to be explored, we can conclude that mutation of the three cysteines in the BST-2 ectodomain did not cause a gross defect in intracellular trafficking.

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