Overexpression of inactive tetherin delGPI mutant inhibits HIV-1 Vpu-mediated antagonism of endogenous tetherin

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A B S T R A C T

Tetherin/BST-2/CD317 inhibits HIV-1 release from infected cells, but the viral Vpu protein efficiently antagonizes this antiviral activity through direct interaction between the transmembrane (TM) domains of each protein. Here, we demonstrated that overexpression of an inactive tetherin delGPI mutant, the TM domain of which could competitively block Vpu targeting of endogenous tetherin, potently inhibited HIV-1 release from human tetherin-positive cells in both transient and stable expression conditions. These results also suggest that heterologous dimerization occurred between the delGPI mutant and endogenous tetherin. These findings suggest that blocking the Vpu/tetherin interface may be a novel therapeutic approach against HIV-1 release.

Structured summary of protein interactions:
- Vpu and Tetherin colocalize by fluorescence microscopy
- Tetherin physically interacts with Vpu by anti tag communoprecipitation

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1. Introduction

HIV-1 has overcome several human defense mechanisms for efficient infection. Thus, blocking viral accessory proteins to restore the antiviral function of host restriction factors is considered a promising approach for HIV-1 treatment. Several recent studies have reported effective inhibitors targeting the antagonist function of HIV-1 Vif against the host protein APOBEC3G [1–3]. Similar to the relationship between APOBEC3G and HIV-1 Vif, the antiviral activity of human tetherin/BST-2/CD317 is counteracted by HIV-1 Vpu [4,5].

Tetherin, an IFN-inducible host restriction factor responsible for the inhibition of HIV-1 release [4,5], contains a transmembrane (TM) domain, a C-terminal glycosylphosphatidylinositol (GPI) anchor [6] and an ectodomain, forming a long roddlike coiled-coil structure [7–10]. The tetherin ectodomain encodes three cysteine residues [11,12], all of which contribute to the formation of cysteine-linked dimers [13,14] and are critical for its antiviral activity [15]. HIV-1 Vpu antagonizes tetherin through a protein–protein interaction which maps to the TM domains of each protein [16–19]. One potential approach to disrupting Vpu function is to target the Vpu–tetherin interface with small molecules or a TM peptide decoy that binds the Vpu membrane-spanning domain and blocks its interactions with tetherin [20]. Although a nuclear magnetic resonance (NMR) structure study has provided information on the interface between the two proteins [21], the crystal structure of the protein complex is not yet available, making the rational design of inhibitors difficult.

In this study, we demonstrated that a human tetherin delGPI mutant retaining the TM domain potently inhibited the release of wild-type HIV-1 in tetherin-positive HeLa cells in both transient and stable expression conditions. However, the corresponding tetherin delGPI mutant containing a replacement African green monkey (AGM) tetherin TM domain showed no such activity, suggesting that this truncated molecule could block and competitively inhibit the Vpu interaction with endogenous tetherin at the cell surface via its TM region. While this molecule also allowed a moderately weak recovery in the release of virus without Vpu, the mutant with changes at the C53,63,91A dimerization sites did not show this activity, suggesting the occurrence of heterologous

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2. Materials and methods

2.1. Plasmid construction

The human tetherin gene (Swiss-Prot entries Q10589) was subcloned into the VR1012 vector for eukaryotic expression. The internally HA-tagged tetherin (inserted in the 3’ end at codon 154) has been reported elsewhere [14,16], and an N-terminally Flag-tagged tetherin was also constructed as previously described [22]. The tetherin mutant lacking the GPI anchor (delGPI) was generated by replacing codon 161 with a termination codon. The human tetherin gene with the TM sequence replaced by the synthetic AGM motif (ShineGene, Shanghai, China) was constructed by overlapping PCR as previously described [16]. The tetherin cysteine-to-alanine mutants were engineered using the QuickChange mutagenesis system (Stratagene, Santa Clara, CA, USA). Fragments encoding tetherin delGPI and the AGM motif delGPI mutant were amplified by PCR and subcloned into an HIV-1-based lentivector vector pLVX-Puro (Clontech, Mountain View, CA, USA) for stable expression. VR1012 encoding codon-optimized HIV-1 NL4-3 Vpu with a cmyc tag and the expression vector for C-terminally HA-tagged human CD4 [19], as well as the HIV-1 proviral clones pNL4-3-3 and pNL4-3 delVpu [23], were previously described.

2.2. Cells and transfections

HeLa (No. CCL-2) and HEK293T (No. CRL-11268) cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA), and the TZM-bl indicator cell line (Catalog No. 8129), was obtained from the National Institutes of Health AIDS Research sas, VA, USA), and the TZM-bl indicator cell line (Catalog No. 8129), was obtained from the National Institutes of Health AIDS Research Evaluation of co-localization of tetherin and Vpu was performed using DAPI (Invitrogen). The samples were observed and imaged on a Zeiss LSM710 confocal microscope.

2.5. Co-immunoprecipitation

Transfected 293T cells were lysed for 30 min at 4 °C in 500 μl lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 (Sigma), 1% CHAPS (Sigma) and a protease inhibitor cocktail (Roche, Basel, Switzerland), followed by centrifugation at 10000 × g for 10 min at 4 °C to pellet the cellular debris. Immunoprecipitations were performed on the pre-cleared supernatants by adding 30 μl HA beads rocking at 4 °C for 4 h. After washing beads five times with lysis buffer, the pellet was resuspended in 30 μl glycine HCl pH 2.0 elution buffer. The eluted samples were separated by SDS–PAGE and analyzed by Western blotting.

2.6. Virion production and infectivity assay

For transient transfection assays, HIV-1 viruses were produced in HeLa or 293T cells in a 6-well plate with 1 μg proviral constructs and indicated amounts of other plasmids. For VSV-G pseudotyped HIV-1 infection assays, 293T cells in a T-25 flask were co-transfected with 3 μg proviral constructs and 500 ng VSV-G plasmids. At 48 h post-transfection, the supernatant was harvested, filtered, and used to infect 293T and HeLa cells in 6-well plates (0.8 ml/well). At 48 h post-infection, cells were washed three times with fresh medium and cultured for 48 h with replacement with 2 ml complete medium. The culture medium of producer cells was clarified with a 0.22-μm filter, and harvest pellets were resuspended in 30 μl RIPA buffer. Virus particle pellets and corresponding cell lysates were analyzed by Western blotting. In single-cycle infectivity assays, 50 μl of the filtered supernatant was mixed with DEAE-dextran (Sigma) at a final concentration of 15 μg/ml and incubated with T2M-bl indicator cells in a 96-well plate. At 48 h post-infection, the cells were lysed, mixed with luciferase substrates and assayed for luciferase activity using a fluorescence microplate reader to represent released virion yield.

2.7. Stable delivery of delGPI tetherin

VSV-G pseudotyped lentiviral particles expressing tetherin mutants were generated by co-transfection of 293T cells with HIV-1 packaging plasmids (RRE, Rev), VSV-G plasmid and the tetherin mutant lentivector. At 48 h post-transfection, the supernatant containing the lentivirus was collected, filtered and used to infect 293T and HeLa cells. Transduced cells were selected for two weeks in complete medium supplemented with 5 μg/ml (293T) or 15 μg/ml (HeLa) puromycin. Expression of stable delGPI tetherin was validated by Western blot using an anti-HA mAb.

3. Results

3.1. Design of tetherin delGPI mutants and their putative activities

The TM domain of tetherin is responsible for Vpu binding, and direct interaction between these molecules is necessary for the dimerization between the delGPI and endogenous tetherin molecules. These findings support the possibility of blocking the Vpu/tetherin interface as a therapeutic approach.
Vpu-induced antagonism of tetherin [24]. We expected that if an inactive tetherin containing the TM domain is still able to bind viral Vpu and block its interaction with endogenous tetherin, it may relieve the inhibitory activity against HIV-1. A previously reported tetherin GPI-anchor deletion (delGPI) mutant can still be transported to the cell surface but is completely devoid of antiviral function [14]. Since that mutant still contains the intact TM domain, we used it along with several other constructs as controls (Fig. 1A). A human tetherin fusion protein, the TM domain of which has been replaced by that of AGM tetherin, is completely insensitive to Vpu [16]. Therefore, the tetherin AGMTM delGPI mutant was introduced to confirm that the potential block of the human delGPI tetherin is mediated by its TM domain.

Three cysteines in the extracellular region (C53, C63 and C91) are responsible for homologous dimerization [13,14]. We hypothesized that if the tetherin delGPI mutant can still dimerize, it may possibly interact with endogenous tetherin. If the heterodimer is formed, it would be of interest to determine the antiviral activity this non-membrane-bound molecule. To verify this hypothesis, we introduced the C53,63,91A mutations into two constructs, tetherin delGPI C-A and tetherin AGMTM delGPI C-A.

3.3. Tetherin delGPI mutant retains dimerization capacity

Reducing/non-reducing conditions were used to detect the aggregation state of tetherin delGPI mutants. Transfected 293T cells were divided into two fractions; one was treated with sample buffer containing β-ME, while the other was treated with sample buffer without β-ME. As shown in Fig. 1C, the reduced samples in the left four lanes show bands corresponding to monomers of ~25 kDa. Among the non-reduced samples in the right four lanes, tetherin delGPI and tetherin AGMTM delGPI migrated as ~50 kDa dimers, while the cysteine mutants, tetherin delGPI C-A and tetherin AGMTM delGPI C-A, showed mostly monomeric bands along with a few dimerization bands. These results suggest that tetherin delGPI could still dimerize, while this ability was significantly impacted by the C53,63,91A mutations.

3.4. Tetherin delGPI mutant partially co-localizes with HIV-1 Vpu

Vpu mainly localizes to the trans-Golgi network (TGN), but it is also found in the endoplasmic reticulum (ER) and the recycling endosome. Vpu and tetherin co-localize in the cytoplasm during the process of tetherin glycosylation in the TGN and transport through the ER and recycling endosomes to the cell surface where it inhibits virus release [4,14,25]. Here, the subcellular localizations of tetherin delGPI and Vpu were evaluated by a confocal immunofluorescence assay. As shown in Fig. 2A, the distribution of tetherin delGPI presented as dense punctas with a tendency to spread outward from the perinuclear area. This pattern indicates that the protein mainly accumulated within the recycling endosome, which is largely consistent with wild-type tetherin [14]. Furthermore, the distribution patterns of tetherin delGPI and Vpu partially overlapped, indicating a certain degree of co-localization. The tetherin AGMTM delGPI showed quite a similar pattern, indicating that replacement of its TM domain may result in loss of sensitivity to Vpu but not from the gross change in subcellular localization.
3.5. Tetherin delGPI mutant directly interacts with HIV-1 Vpu

Next, the interaction of delGPI tetherin with Vpu was verified by immunoprecipitation. 293T cells were co-transfected with Vpu-cmyc and tetherin delGPI or AGMTM delGPI plasmids. Cells were lysed and incubated with HA-beads to precipitate HA-tagged tetherin. As shown in Fig. 2B, expression levels of Vpu in the three samples were comparable, and those of delGPI and AGMTM delGPI tetherin were similar as well (pre-immunoprecipitation lysates). Vpu was nearly absent in the mock control immunoprecipitation sample. Meanwhile, Vpu was pulled down with tetherin delGPI. However, when tetherin AGMTM delGPI was precipitated, the Vpu band was much weaker than that with tetherin delGPI. This result confirmed that the tetherin delGPI mutant maintained Vpu binding activity.

3.6. Tetherin delGPI mutant inhibits Vpu-induced degradation of tetherin and CD4

Down-regulation of tetherin and CD4 are important Vpu functions, both of which involve the decrease of the target protein at the cell surface and subsequent cellular degradation. Currently, the tetherin interacting region is considered to be located mainly in the Vpu TM domain; meanwhile, the cytoplasmic domain of Vpu are critical for CD4 interaction, although the Vpu TM domain has also been implicated in this process [26]. We have also previously shown that mutation in the C-terminal end of the Vpu TM domain affects Vpu-induced CD4 down-regulation [19]. After demonstrating the considerable affinity of delGPI tetherin for Vpu, we carried out co-transfection experiments in 293T cells to further validate its influence on Vpu functions. As shown in Fig. 2C, delGPI tetherin exhibited no significant effect on the level of wild-type tetherin in the absence of Vpu. In the presence of Vpu, however, delGPI tetherin drastically altered the Vpu-induced degradation of wild-type tetherin, while its counterpart AGMTM delGPI tetherin could not. Unexpectedly, delGPI tetherin also inhibited CD4 degradation as shown in Fig. 2D, suggesting a considerably potent block directed toward the whole Vpu TM domain. Another intriguing observation was that delGPI tetherin itself was resistant to the Vpu-induced degradation even though it could strongly interact with Vpu.

3.7. Transient expression of tetherin delGPI mutant inhibits HIV-1 virion release in tetherin-positive cells

In order to detect its blocking effect on Vpu, the tetherin delGPI mutant was co-expressed with a wild-type or Vpu-defective HIV-1 provirus in endogenous tetherin-positive HeLa cells, followed by assessment of virion release. Relatively high doses of vectors were used in the transfections. Virions in the supernatants were pelleted to analyze the p24 capsid protein content, while cells were collected for the detection of Pr55Gag and tubulin. As shown in Fig. 3A (left panel, wild-type HIV-1; right panel, Vpu-defective HIV-1), Pr55Gag levels in all samples were equal. Compared to the release of wild-type virus which was set to 100% (Fig. 3A, lane 1), the release of wild-type virus from cells expressing tetherin delGPI decreased to about 1/3 (Fig. 3A, lane 2), while it was essentially unchanged from cells expressing tetherin AGMTM delGPI (Fig. 3A, lane 3).
lane 3). The two mutants containing the C53,63,91A changes showed similar inhibitory effects on the wild-type virus (Fig. 3A, lanes 4 and 5). Meanwhile, the release of Vpu-defective HIV-1 was relatively low (Fig. 3A, lane 6). In the presence of the tetherin delGPI mutant, the release of Vpu-defective virus was still significantly inhibited, but it was moderately higher than that in the absence of tetherin delGPI mutant (Fig. 3A, lane 7). Of note, levels of virus release with tetherin delGPI and tetherin AGMTM delGPI (Fig. 3A, lanes 7 and 8) were even higher than those in the presence of the two C53,63,91A cysteine mutants (Fig. 3A, lanes 9 and 10). These observations suggest that overexpression of the TM domain containing tetherin mutant in tetherin-positive HeLa cells resulted in considerable inhibition of the release of Vpu-positive HIV-1.

3.8. Transient expression of tetherin delGPI mutant dose-dependently inhibits infectious HIV-1 release in tetherin-positive cells

To further validate the above results, we carried out a similar but more elaborate virus release experiment with a range of doses of tetherin delGPI plasmids. Here we measured virion release with a high-throughput assay by titration of infectious particles on TZM-bl indicator cells. The culture supernatants of transfected cells were collected, filtered and used to infect TZM-bl cells, which were cultured for another 48 h before luciferase activity detection. The luciferase values were converted to percentages, and the wild-type virus release without any tetherin plasmid was set to 100% (Fig. 3B). The release of wild-type as well as Vpu-defective HIV-1 gradually decreased with increasing amounts of the fully functional wild-type tetherin used as a positive control (Fig. 3B, a). With gradually increasing amounts of tetherin delGPI and tetherin delGPI C-A (Fig. 3B, b and d, hollow points), the release of the wild-type HIV-1 displayed a similar declining trend as with the wild-type tetherin; whereas the corresponding AGMTM mutants showed no significant effects on the release of wild-type HIV-1 (Fig. 3B, c and e, hollow points). On the other hand, increases of tetherin delGPI and tetherin AGMTM delGPI resulted in a moderately weak increase of the release of Vpu-defective HIV-1 (Fig. 3B, b and c, solid points), while their corresponding C53,63,91A mutants did not.
not induce this phenomenon (Fig. 3B, d and e, solid points). From these dose–response experiments, we saw basically the same trend as with the above overexpression experiments in that the TM domain containing tetherin delGPI mutant could block the interaction between HIV-1 Vpu and endogenous tetherin with relatively high efficiency.

3.9. Stable delivery of tetherin delGPI mutant inhibits infectious HIV-1 release in tetherin-positive cells

Whether an antiviral molecule can be practically expressed in sufficient amounts in a clinically relevant context and whether it exhibits comparable effects in a steady state of expression and in transient expression are important determinants of success of the therapeutic strategy. After observing above the potent inhibition of Vpu by transiently expressed delGPI tetherin protein, tetherin-positive HeLa cells and tetherin-negative 293T were used to stably express delGPI tetherin (with AGMTM delGPI tetherin as a control) via a lentiviral vector as described in Materials and Methods. After two weeks of lentivirus infection and antibiotic screening, cells expressing the protein of interest in a steady state were obtained and confirmed by Western blotting. The expression pattern of the stable tetherin delGPI protein, similar to endogenous tetherin, showed more multimeric bands than did the transient tetherin protein. (Fig. 4A). Additionally, the expression level of the mutant in 293T cells was slightly higher than that in HeLa cells (Fig. 4A). The different types of cells were infected with VSV-G pseudotyped HIV-1. The released virions were evaluated by ultracentrifugation, and infectious particles were titrated on TZM-bl indicator cells. As shown in Fig. 4A and B, nearly the same levels of wild-type HIV-1 and Vpu-defective viruses were released from 293T cells, and no significant difference was observed in the levels of released virions from the parental 293T cells and 293T cells stably expressing tetherin mutant. In the parental HeLa cell line, HIV-1 delVpu was released at a low level, but wild-type HIV-1 was still released at a high level. Notably, the level of released wild-type HIV-1 decreased by more than 60% in HeLa cells stably expressing delGPI tetherin, while no such apparent change was observed in HeLa cells stably expressing AGMTM delGPI tetherin. In addition, the stable delGPI and AGMTM delGPI tetherin also showed a very weak up-regulatory effect on release of Vpu-defective HIV-1.

4. Discussion

Further improvements in treatment options would be best obtained by in-depth studies of the virological characteristics and pathogenic mechanisms of HIV-1 in order to discover new drug targets. Induction of the tetherin host restriction factor by IFN-α [27] to enhance its antiviral activity or inhibition of its antagonist HIV-1 Vpu are both potential options for the treatment of HIV-1. However, due to the involvement of IFNs in many other biological processes, the non-specific IFN-α treatment may cause many side effects which would not render it a particularly feasible therapeutic approach [28,29]. A functionally complete tetherin TM variant will probably be a more potent alternative. However, an excess of a functional antiviral protein is likely to trigger other unknown effects, such as the heavy selective pressure that may cause the virus to evolve new a mechanism to antagonize or evade the inhibition. Notably, the effect of tetherin on HIV-1 has been considered recently to extend beyond the inhibition of virion release [reviewed in [30]]. Other functions proposed for tetherin have included the enhancement [31] or inhibition [32] of cell-to-cell transmission, and even the decrease of viral infectivity [33]. Although the precise tetherin functions especially in the lymphocytes are still in debate, some of these observed phenomena may be attributed to the use of a strong promoter to express exogenous functional tetherin. Therefore, restoring the function of endogenous host antiviral factor would be the best therapeutic weapon, as well as a safer and more effective antiviral strategy, if inhibiting the viral antagonistic factor could be achieved.

To date, the mechanism of Vpu-mediated antagonism of tetherin has not been fully defined, making it difficult to find methods/agents to inhibit this viral factor. Amphotericin B methyl ester (AME) was found to interfere with the HIV-1 budding process in a Vpu-dependent manner [34], suggesting that it may disrupt the anti-tetherin function of Vpu. However, the blocking mechanism of AME against Vpu activity has not been further elaborated.

In conclusion, we have demonstrated that the tetherin delGPI mutant potently inhibited the release of wild-type HIV-1 from tetherin-positive HeLa cells, suggesting that this truncated molecule could block and competitively inhibit the interaction of Vpu with endogenous tetherin through its TM region. Meanwhile, the tetherin delGPI mutant also caused a moderately weak recovery of virus release in the absence of Vpu, raising the possibility of heterologous dimerization between this molecule and endogenous tetherin. These results support the feasibility of blocking the Vpu/tetherin interface as a therapeutic target. Specifically, interrupting the interaction between HIV-1 Vpu and endogenous tetherin via gene transfer of tetherin TM helical peptides or small molecule inhibitors may be a novel and feasible approach for future treatments aimed at inhibition of HIV-1 release.
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