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Purification of eukaryotic tetherin/Vpu proteins and detection of their interaction by ELISA

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ABSTRACT

Tetherin/BST-2/CD317 inhibits HIV-1 release from infected cells, while HIV-1 Vpu efficiently antagonizes tetherin based on intermolecular interactions between the transmembrane domains of each protein. In this study, we successfully partially purified His-tagged tetherin with a glycophosphatidylinositol deletion (delGPI) and His-tagged full-length Vpu from transiently transfected 293T cells using affinity chromatography. The *in vitro* interaction between these purified proteins was observed by a pull-down assay and ELISA. Detection of the Vpu/tetherin interaction by ELISA is a novel approach that would be advantageous for inhibitor screening *in vitro*. Successful co-purification of the tetherin/Vpu complex also provides a basis for further structural studies.

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Introduction

Tetherin/BST-2/CD317 is an interferon-inducible cellular factor which blocks the release of HIV-1 particles from infected cells [1,2]. It is a 30–36 kDa type II single-pass transmembrane (TM)[3] protein [3] that encodes an N-terminal cytoplasmic tail, followed by a TM domain, an extracellular coiled–coil domain and a putative C-terminal glycophosphatidylinositol (GPI)-linked lipid anchor [4]. The unusual topology enables tetherin to directly tether newly forming HIV-1 particles and other enveloped viruses to the host cell surface and thus inhibit virion release [1]. However, HIV-1 expresses accessory proteins to counteract such host defenses [5]. The tetherin-induced restriction of HIV-1 release is neutralized by Vpu [1,2], which is a 18 kDa type-I TM protein encoded by HIV-1 [6,7]. It is comprised of a TM domain and a cytoplasmic domain [8]. The direct interaction between Vpu and tetherin via their respective TM domains is critical for the function of Vpu in overcoming tetherin [9,10].

The TM nature of Vpu and tetherin, as well as the glycosylation in the extracellular domain of tetherin, makes it difficult to separate and purify these proteins in a prokaryotic expression system. Although the heterologous overexpression of tetherin has been extensively explored, only limited progress has been achieved, and only the crystal structure of the extracellular domain of tetherin has been reported [11–13]. Recently, an anti-parallel helix-helix packing model of the interaction between the TM domain of Vpu and BST-2 was developed using nuclear magnetic resonance (NMR) spectroscopy [14]. However, the crystal structure of the Vpu/tetherin protein complex remains to be determined. Very recently, we reported that the overexpression of an inactive tetherin delGPI mutant, the TM domain of which competitively blocks Vpu targeting tetherin, potently restores the function of endogenous tetherin to inhibit HIV-1 release from human tetherin-positive cells both in transient and stable expression conditions [15]. This previous study suggested that blocking the Vpu/tetherin interface may be a novel therapeutic approach against HIV-1 release. Therefore, we perform the following study to further investigate *in vitro* interaction of GPI-truncated tetherin and wild-type vpu, trying to develop a high throughput method for the screening of candidate inhibitor of Vpu/tetherin interaction. We added a 6× His tag to the C-terminus of GPI-truncated tetherin or Vpu for affinity purification. Following transient expression in 293T cells, the proteins were partially purified using nickel affinity chromatography. Thereafter, we analyzed the direct protein–protein interaction between Vpu and tetherin *in vitro* by using a pull-down assay and ELISA, the results of which suggested that the purified proteins

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[3] *Abbreviations used:* TM, transmembrane; GPI, glycophosphatidylinositol; NMR, nuclear magnetic resonance; DMEM, Dulbecco's modified Eagle's medium; PEI, polyethylenimine; Ni-NTA, nickel-nitrilotriacetic.

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are functional. Detection of the Vpu/tetherin interaction by ELISA is a novel approach that may be used for *in vitro* inhibitor screening. Additionally, the successful co-purification of eukaryotic Vpu and tetherin also provides a foundation for future studies on the crystal structure of these proteins in complex.

Materials and methods

Plasmid construction

The human tetherin gene (Swiss-Prot entries Q10589) was subcloned into the VR1012 vector for eukaryotic expression as previously described [16]. Tetherin delGPI H6 is a tetherin mutant lacking the GPI anchor, which was generated by inserting a 6× His tag in frame after codon 159, followed by a stop codon. Using this construct, residues 1–50 residues were removed to generate tetherin delGPI/TM H6. VR1012 encoding the codon-optimized HIV-1 NL4-3 Vpu sequence with a 6× His tag (or cmyc tag) was derived by PCR from pcDNA-Vphu provided by the National Institutes of Health AIDS Research and Reference Reagent Program (NIH-ARRRP, Germantown, MD, USA), and a 6× His tag (or cmyc tag) was added in frame at its C-terminus. Vpu TM MΔ3I, a tetherin insensitive Vpu mutant with a 3 isoleucine deletion in its TM domain, was described previously [16].

Cell culture and transfections

HEK293T (No. CRL-11268) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). For batch transfection, cells were cultured in ten T125 flasks and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) at 37 °C/5% CO_2. Cells were transfected with the indicated construct(s) using polyethylenimine (PEI, Sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography

Approximately 48 h after transfection, cells were harvested in PBS and centrifuged at 800*g* for 10 min. The cell pellet (about 0.70 g wet weight from a flask) from ten T125 flasks was resuspended in 20 ml cell lysis buffer containing 150 mM NaCl, 50 mM Tris–Cl, 0.5% Triton X-100 (Sigma), 5% glycerol and a protease inhibitor cocktail (Roche, Basel, Switzerland). The cell suspension was sonicated for 10 min on ice and then centrifuged at 16,000*g* for 30 min. The supernatants were applied to 1 ml of Probond nickel resin (Invitrogen, Carlsbad, CA, USA). To minimize non-specific binding, 5 mM imidazole was added to the supernatant during this step. The column was washed with 10 bed volumes of wash buffer containing 150 mM NaCl, 50 mM Tris–Cl, 5% glycerol and increasing concentrations of imidazole (10, 20, 35 and 50 mM). The protein was eluted with 10 bed volumes of wash buffer containing 500 mM imidazole and concentrated with a 10 kDa ultrafiltration tube (Millipore, Billerica, MA, USA) to the final volume of approximately 500 μl. The elution profile was monitored by measuring the absorbance at 280 nm.

Protein analysis

Samples collected from each step of the experiments were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized with Coomassie Brilliant Blue staining (Bio-Rad, Hercules, CA, USA). For detection of proteins by Western blotting, samples were resolved by SDS–PAGE and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Tetherin delGPI H6 was detected with a mouse monoclonal anti-His antibody (Covance, Princeton, NJ, USA) or anti-BST2 monoclonal IgG1 antibody (Abnova, Taipei, Taiwan). Vpu H6 was detected with a mouse monoclonal anti-His antibody (Covance). Untagged Vpu was detected with rabbit polyclonal anti-Vpu serum (NIH-ARRRP). In the pull-down assay, Vpu cmyc was detected with a mouse monoclonal anti-myc antibody (Millipore). Alkaline phosphatase conjugated goat anti-mouse and goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) were used as secondary antibodies. The purified protein concentration was determined by absorbance measurements at 280 nm using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The extinction coefficients are 0.321 g/L for tetherin delGPI H6 and 1.253 g/L for Vpu H6, as determined by the ProtParam (http://web.expasy.org). Total protein for each major purification steps was measured by the Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). The purity of each protein was estimated by densitometric measures of the proteins on SDS–PAGE using Quantity One software (BioRad, Hercules, CA, USA).

Peptide-N-Glycosidase F (PNGase F) digestion

Proteins were incubated in glycoprotein denaturing buffer at 95 °C for 15 min. NP-40, G7 reaction buffer and PNGase F (New England Biolabs, Ipswich, MA, USA) were added and further incubated for 1 h at 37 °C. Subsequently, samples were mixed with SDS sample buffer and analyzed by SDS–PAGE and Western blotting.

Pull-down assay

The purified tetherin delGPI H6 protein was incubated with Probond nickel resin for 1 h at 4 °C. 293T cells transfected with the Vpu-cmyc expression construct for 48 h were harvested and lysed, and the clarified supernatant was incubated with the tetherin delGPI H6 bound resin for 2 h at 4 °C. After washing with 5 ml wash buffer with an increasing gradient of imidazole (10, 20, 35 and 50 mM), bound proteins were eluted and analyzed by SDS–PAGE and Western blotting.

ELISA

Ninety-six-well microtiter plates were coated with Vpu H6 (1 μg/ml) in a coating buffer composed of 15 mM Na_2CO_3 and 35 mM $NaHCO_3$. After overnight incubation, the plates were washed three times with 200 μl of wash buffer (PBS containing 0.05% Tween 20). The plates were then incubated with blocking buffer containing 3% bovine serum albumin for 2 h at 37 °C. After three washes, serial 2-fold dilutions of tetherin delGPI H6 and tetherin delGPI/TM H6 in blocking buffer were prepared, and 100 μl of each dilution was added per well. After 90 min of incubation at 37 °C, the wells were washed six times and then incubated with 100 μl of diluted anti-BST2 monoclonal IgG1 antibody (Abnova) for 2 h at 37 °C. The wells were washed three times, and the horseradish peroxidase (HRP)-coupled goat anti-mouse antibody (Jackson Immunoresearch) at a 1:10,000 dilution in blocking buffer was applied for 1 h. Finally, the plates were washed three times and developed with 100 μl of TMB reagent (Tiangen, Beijing, China) for 30 min before the reaction was terminated by the addition of 100 μl of 2 M H_2SO_4. Optical densities (OD) at 450 nm were determined using a microplate reader (Bio-Rad).

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