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Inhibition of HIV Budding by a Genetically Selected Cyclic Peptide Targeting the Gag–TSG101 Interaction

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ABSTRACT The egress of HIV particles from virus-infected cells is accomplished by the recruitment of proteins that normally mediate host cell endocytic functions. This process requires interaction of the HIV Gag protein with the host protein TSG101 (tumor susceptibility gene 101). Here, we report the use of a bacterial reverse two-hybrid system to identify cyclic peptides that interfere with the Gag–TSG101 interaction and the finding that a five amino acid peptide discovered by this approach can disrupt the interaction and consequently inhibit HIV egress. The inhibiting molecule, which was selected from a cyclic peptide library containing $\sim 3.2 \times 10^6$ members, differs in primary sequence from the interacting sites of either TSG101 or Gag. Addition of cyclic peptide tagged with an HIV Tat sequence, which previously has been shown to enhance protein translocation across plasma membranes, to cultured human cells inhibited the production of virus-like particles (VLPs) by these cells (IC_{50} of 7 μM), and this inhibition occurred in the absence of adverse affects on normal endocytic functions mediated by TSG101. A mutant Gag protein not dependent on TSG101 for release was unaffected by the cyclic peptide. Our findings, which suggest that interference with the TSG101–Gag interaction by cyclic peptides may be of practical use in the treatment of HIV infections, identify a specific cyclic peptide that reduces VLP release by this mechanism; they also demonstrate that the efficiency of interference with protein–protein interactions by cyclic peptides can be enhanced by tagging the peptides with translocation-promoting sequences. Collectively our results support the notion that small molecule therapeutics that inhibit specific interactions between viral and host proteins may have general applicability in antiviral therapy.

The poor fidelity of HIV genome replication is advantageous to the virus, allowing the evolution of mutant forms resistant to therapeutic agents. About 50% of infected individuals are believed to harbor drug-resistant strains of HIV (*1*), highlighting the need for new therapeutic strategies (*2*). HIV recruits host cell proteins to mediate functions necessary for viral propagation (for review, see refs 3 and 4. Such host proteins offer points of possible antiviral interventions that are alternatives to therapies aimed directly at the virus and thus may be less subject to circumvention by viral mutation (*4*).

To complete the viral life cycle, HIV particles assemble on and subsequently are released from the plasma membrane of infected cells (*5, 6*). Release of the virus requires the virally encoded Gag protein (*7, 8*), plus the active participation of multiple host proteins (*5, 9*). The p6 region of Gag, known as the late budding domain, contains a highly conserved P(T/S)AP tetrapeptide motif that is essential for the viral budding event (*10, 11*). This motif specifically interacts with the UEV (ubiquitin E2 variant) domain of the host protein TSG101 (*12–14*). TSG101, which was identified initially as a tumor susceptibility gene protein (*15*), is a central component of the endocytic machinery (*16*) that normally binds to the endosomal protein HRS (hepatocyte growth factor-regulated tyrosine kinase substrate), through a PSAP motif (*17, 18*). By mimicking the binding of HRS through its own PTAP motif, HIV Gag recruits TSG101 and associated endocytic complex proteins to the plasma membrane to mediate viral release (*19*). Normal cellular levels of TSG101 are required for the budding of HIV, as inhibition of TSG101 synthesis or overexpression of the gene severely impairs virus production

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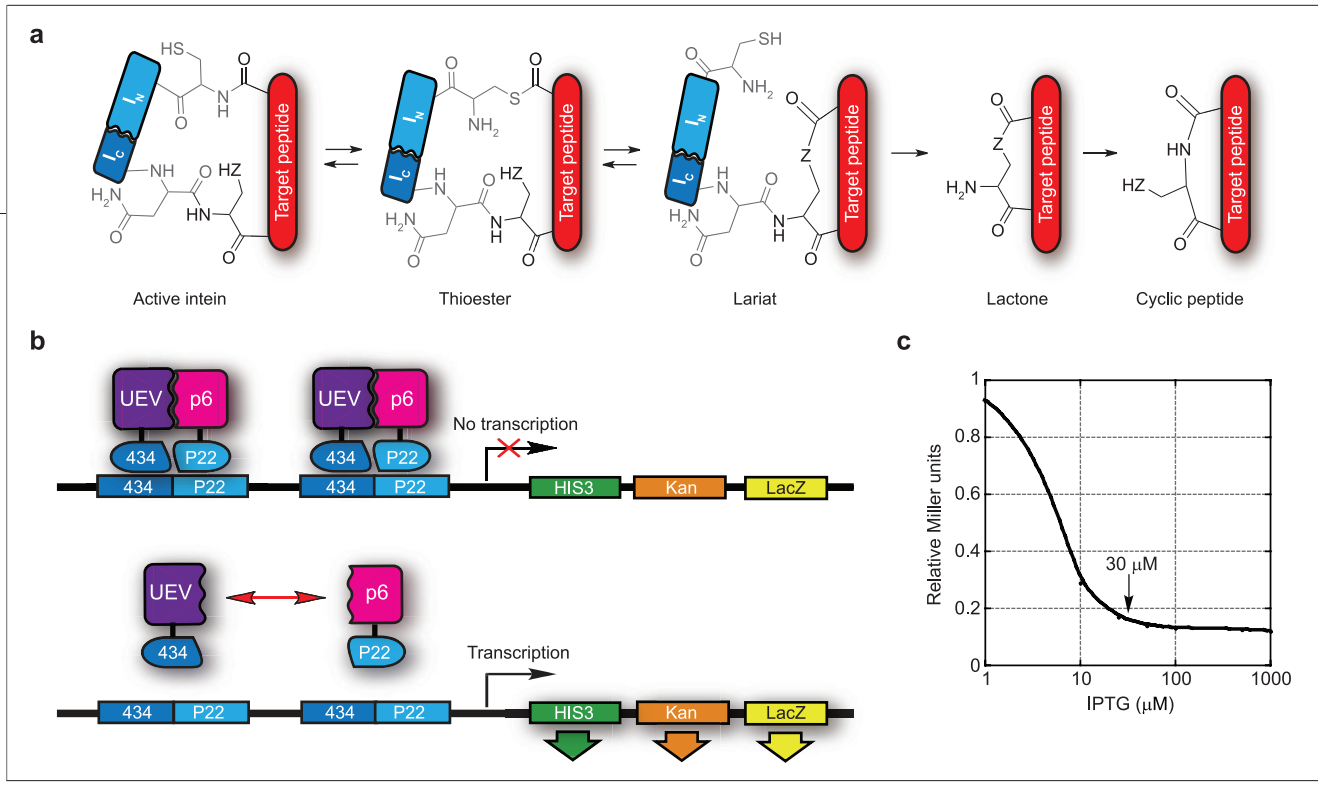

Figure 1. Schematic summary of the procedures used for cyclic peptide production and reverse two-hybrid screening. a) The SICLOPPS plasmid codes for a split intein that folds to form an active intein, which undergoes a series of rearrangements to generate a cyclic peptide. In this case each SICLOPPS plasmid codes for a target cyclic peptide with randomly encoded amino acids forming libraries of ~108 members. Z = S or O (cysteine or serine). b) Bacterial reverse two-hybrid system. Top panel: The target proteins are expressed as fusions with the 434 or P22.434 bacteriophage DNA binding proteins, which associate to form a functional repressor that prevents expression of the reporter genes (inhibiting growth on minimal media). Bottom panel: A small molecule inhibits the protein–protein interaction of the target proteins (p6 and UEV), allowing transcription and translation of the reporter genes that rescue growth by induction of HIS3 and Kan^R. β-Galactosidase assays were used to quantify repression of *lacZ* expression. c) ONPG assay of the p6–UEV reverse two-hybrid system. The data shows increasing IPTG levels (increased expression of the fusion proteins that dimerize to form a functional repressor) resulting in reduced activity of the LacZ reporter gene product (β-galactosidase), as quantified by monitoring the cleavage of ONPG. This confirms the dimerization of UEV and p6 in our reverse two-hybrid system. The optimal IPTG level for genetic selection was determined to be 30 mM, the minimum level at which intracellular fusion protein concentration is sufficient to form a functional repressor.

by arresting the release of viral particles from the plasma membrane of host cells (*12, 20*). Furthermore, mutations in motifs of either TSG101 or Gag that are essential for the interaction of these proteins also reduce the production of infectious viral particles (*12, 13*). The Gag–TSG101 interaction thus offers a potential target for antiviral therapy. Recently, Burke and colleagues (*21, 22*) have generated chemically modified PTAP-containing peptides with enhanced affinity for TSG101 in an effort to develop inhibitors of Gag–TSG101 interaction.

A previously reported method enables genetic selection of cyclic peptide inhibitors of protein–protein interactions; the methodology combines SICLOPPS (split intein-mediated circular ligation of peptides and proteins) libraries (*23, 24*) with a bacterial RTHS (reverse two-hybrid system). SICLOPPS is a robust and flexible method for the biosynthesis of small molecule libraries; a plasmid library is constructed (with degenerate oligonucleotides) coding for the C- and N-terminus of the *Synechocystis* sp. PCC6803 DnaE split inteins, joined by a predetermined number of random (or intermittently random) amino acids. After intracellular expression, the split inteins process to give a cyclic peptide (Figure 1, panel a). The cyclic peptide library can contain up to a hundred million (10^8) members (*25, 26*). The bacterial RTHS (*25, 26*) is based on the bacteriophage regulatory system, using chimeric repressor fusions and promoter sequences to link the disruption of targeted fusion protein heterodimers to the expression of three reporter genes. HIS3 (imidazole glycerol phosphate dehydratase) and Kan^R (aminoglycoside 3′-phosphotransferase for kanamycin resistance) are two chemically tuneable, conditionally selective reporter genes. The third reporter gene, LacZ (β-galactosidase), is used to quantify the protein–protein interaction through β-galactosidase assays. The combined SICLOPPS/RTHS system thus can identify cyclic peptide inhibitors and concurrently reveal the relative affinity and selectivity of each inhibitor.

We used SICLOPPS/RTHS to identify novel cyclic peptides that disrupt the Gag–TSG101 interaction. Here

we report that an eight-member cyclic peptide that had similar effects on disruption of the Gag—TSG101 and HRS—TSG101 interaction inhibited release of virus-like particles (VLPs) when translocated into VLP-producing cells, while still allowing normal endocytosis. Our results provide evidence for the therapeutic potential of small molecule interference with virus-recruited host protein functions.

RESULTS AND DISCUSSION

Since the p6 region of HIV Gag and the UEV domain of TSG101 are both necessary and sufficient for the Gag—TSG101 interaction, we introduced DNA fragments encoding the p6 region of Gag and the UEV domain of TSG101 into the RTHS plasmid and cloned these constructs in *E. coli*. Expression of the gene fragments was under the control of an isopropyl β-D-thiogalactoside (IPTG) inducible promoter (Figure 1, panel b). To eliminate false selections arising from plasmid ejection, the cassette coding for the p6-P22.434 and UEV-434 repressor fusion proteins was integrated into the chromosome of an *E. coli* reporter strain by previously reported methods (*26, 27*). Upon chromosomal integration of the p6—UEV reverse two-hybrid system, *o*-nitrophenyl-β-galactoside (ONPG) assays were carried out to measure the IPTG-dependent repression of the LacZ gene product (β-galactosidase). Increased IPTG-dependent expression of the fusion proteins resulted in decreased β-galactosidase activity (Figure 1, panel c), indicating that UEV and p6 interact in our reverse two-hybrid system.

To identify cyclic peptides that may inhibit the p6—UEV interaction, we constructed a SICLOPPS library using oligonucleotides that encode for cyclic peptides of type SGWXXXXX (X = any amino acid). The invariable motif of the peptide was designed to contain serine (a required nucleophile for intein processing), glycine (to avoid racemization during chemical synthesis), and tryptophan, which functions as a chromophore for HPLC purification. After electroporation of the SICLOPPS plasmid library into *E. coli* (DH5α), we obtained ~4×10^8 colonies, giving good coverage of our cyclic peptide library (3.2×10^6 six-member cyclic peptides, with five random residues). Isolated plasmids from this library were transformed into the selection strain (1×10^8 transformants) and plated onto histidine-free minimal media at a density of 10^6 per plate (100 mm × 15 mm). The selection plates were supplemented with arabinose (inducer for SICLOPPS), 3-amino-1,2,4-triazole (3-AT, competitive inhibitor of HIS3 gene product), and kanamycin. The plates were incubated until colonies were readily visible (~1 in 10^5). Approximately 100 colonies were picked and screened for IPTG-dependent growth inhibition and arabinose-dependent growth advantage in order to eliminate false positives. The expected phenotype was further confirmed by isolating and retransforming the selected SICLOPPS plasmids into the p6—UEV selection strain. After several rounds of secondary screening, we identified four cyclic peptides that potentially inhibit the UEV—p6 interaction (Table 1).

TABLE 1. Sequence of the genetically selected cyclic peptide inhibitors of p6—UEV dimerization, in order of biological activity

Activity rank	Name	Peptide sequence
1	11	I Y W N V SGW
1	6	T N W Y G SGW
2	16	T L L V Y SGW
3	8	V L R V H SGW
3	120	P G P V T P G F SGW
3	112	D G P R G P S T SGW
3	127	P G P C S P V G SGW
3	126	L V P W M P R P SGW
3	119	G C P F P P S Y SGW
3	122	A R P N R P C R SGW

It is noteworthy that none of the identified cyclic peptides has a primary sequence resembling P(S/T)AP. However, as the Gag—TSG101 interaction is known to be mediated by PTAP (*28*), we also constructed a SICLOPPS library that mimicked this motif (SGWXXPXXPXX). The new library was introduced by transformation into the p6—UEV selection strain (10^8 transformants). Six colonies were identified after two rounds of screening. The cyclic peptide inhibitors (from both libraries) were ranked for their activity to confer growth advantage by spotting 10-fold serial dilutions of the corresponding cells onto selective media (Table 1).

There is a certain degree of homology among the amino acid sequences of the three cyclic peptides that showed the greatest inhibition. Threonine is favored at

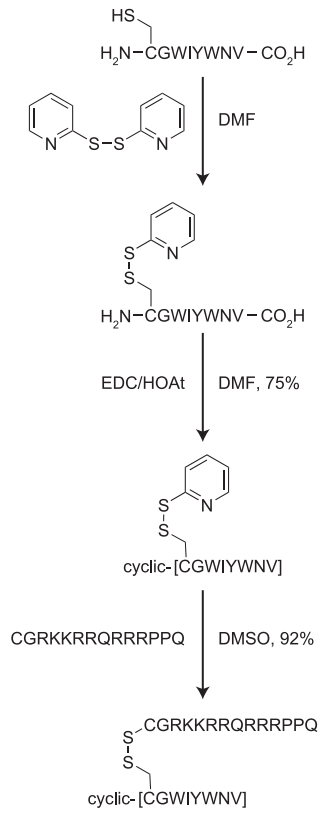

Figure 2. Tagging the genetically selected cyclic peptide 11 with a cysteine-modified HIV Tat peptide. The cysteine of the corresponding linear peptide was protected with 4,4′-dithiodipyridine (Aldrithiol), and the peptide was cyclized as previously described. After purification by ether precipitation, cysteine-modified Tat peptide was added at 2-fold excess to the cyclic peptide in DMSO solution. The resulting Tat-tagged cyclic peptide was purified by HPLC, and its identity was confirmed by MALDI MS. This process was repeated for peptides 6 and 16.

position one and tryptophan at position three. An interesting occurrence is the presence of the NWY motif in the top two peptides (but in reverse order in the second peptide), suggesting that the same structural feature is being targeted by both inhibitors. The inhibitory cyclic peptides obtained from the PTAP targeted library were not studied further, as they were less active as inhibitors than those from the fully randomized library. This finding, which argues that the cyclic peptide does not act simply as a peptomimetic, suggests that the mechanism underlying the disruption of the TSG101–Gag interaction is likely to be conformationally based. To assess the specificity of the genetically selected cyclic peptides for the p6–UEV interaction, the corresponding SICLOPPS plasmids were introduced into a RTHS strain used previously to select for inhibitors of the large and small subunits of murine ribonucleotide reductase (RR) (*25*). Specific p6–UEV inhibitors are expected to be inactive in the RR strain, which was identical to the p6–UEV selection strain except for the target protein dimer. Three of the four selected peptides (peptides 6, 11, and 16) did not confer a growth advantage (arabinose-dependent) on the RR strain and were therefore considered likely to be specific inhibitors for the p6–UEV interaction.

As HIV budding is dependent on the protein–protein interaction between TSG101 and Gag, we turned our attention to assaying the cyclic peptide inhibitor's ability to interfere with HIV budding in mammalian cells. To this end, cyclic peptides 6, 11, and 16 were chemically synthesized and purified using previously described solid-phase chemistry (*26*). The synthetic peptides were identical to the corresponding biologically produced SICLOPPS peptides, as shown by both reverse-phase HPLC and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.

We used a Gag VLP (viral-like particle) assay to monitor the effects of the cyclic peptides on viral budding. The assay is based on the finding that HIV Gag protein itself is sufficient to bud from the cell surface and give rise to VLPs that are morphologically similar to *bona fide* virus particles (*6, 8, 29*). Human embryonic kidney 293T cells were transfected with a Gag-GFP fusion construct (*30*) and incubated in media containing one of the three cyclic peptides (10 μM). Forty-eight hours after transfection initiation, cell lysates and viral-like particles were collected and analyzed by Western blotting.

Notwithstanding the observed ability of cyclic peptides generated intracellularly to interfere with interaction between TSG101 and Gag, we observed no effects of the peptides on Gag-mediated VLP release when the same peptides were added to culture media at concentrations up to 25 μM. We hypothesized that this finding could be due to the failure of externally administered cyclic peptides to enter cells in sufficient quantity to significantly affect TSG101–Gag binding and subsequently inhibit Gag egress. To improve cyclic peptide delivery into targeted mammalian cells, we attached a short peptide sequence derived from the HIV Tat inter-

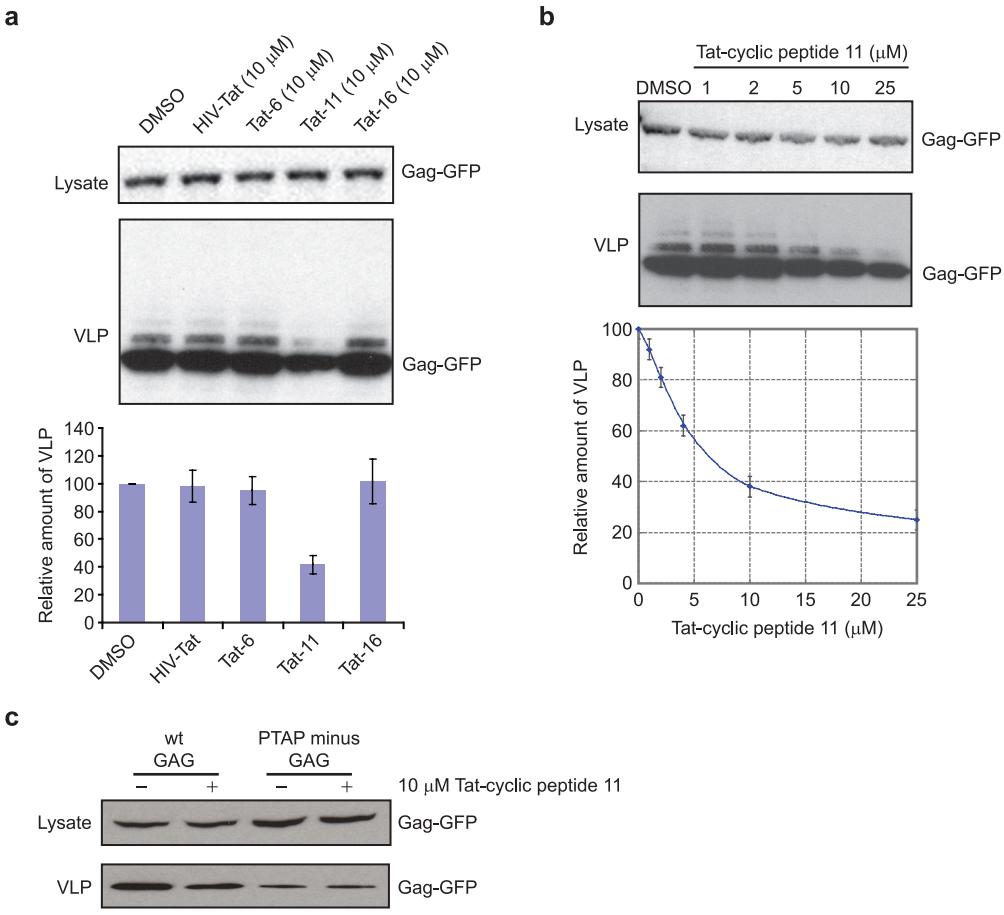

Figure 3. Gag VLP release assay. a) The effect of the Tat-tagged cyclic peptides on the level of Gag-GFP protein in the lysate and in virus like particles (correlated to VLP release). Peptide 11 reduces VLP release by 60% at 10 μM. DMSO and the HIV Tat sequence by itself were tested as negative controls. b) Effect of Tat-tagged cyclic peptide 11 on VLP release (and the level of Gag-GFP in the lysate) at various concentrations. The IC_{50} of peptide 11 was determined to be 7 μM by this method. c) Effect of Tat-tagged cyclic peptide 11 on VLP release of wild-type and PTAP mutant Gag constructs.

nalization domain; the synthesized Tat sequence, which previously has been shown to aid the translocation of a variety of proteins across the plasma membrane of mammalian cells (*7, 11*), was attached to the cyclic peptide through a disulfide bond between two cysteines. This had been formed by modifying the nonrandom region of the cyclic peptide from SGW to CGW and by adding a cysteine to the beginning of Tat (Figure 2).

Unlike its untagged parent, Tat-tagged cyclic peptide 11 at a 10 μM concentration inhibited VLP release by >60% upon addition to cultures of 293T cells used in the Gag-VLP assay. The same concentration of Tat-tagged cyclic peptides 6 and 16 or of the Tat sequence alone had no effect on VLP release (Figure 3, panel a). The inhibitory effect of cyclic peptide 11 on VLP production was found to be dose-dependent, with an IC_{50} of 7 μM (Figure 3, panel b). The dependence of the cyclic peptide's inhibition of VLP production on the TSG101 interaction was tested using a mutant Gag protein that lacks a PTAP motif (*12*) but still can be released as VLP, although to a much lower extent than wild-type Gag (ref 12; Figure 3, panel c). Whereas addition of Tat-cyclic peptide 11 reduced the release of wild-type Gag as VLP, we observed no effect of the peptide on release of

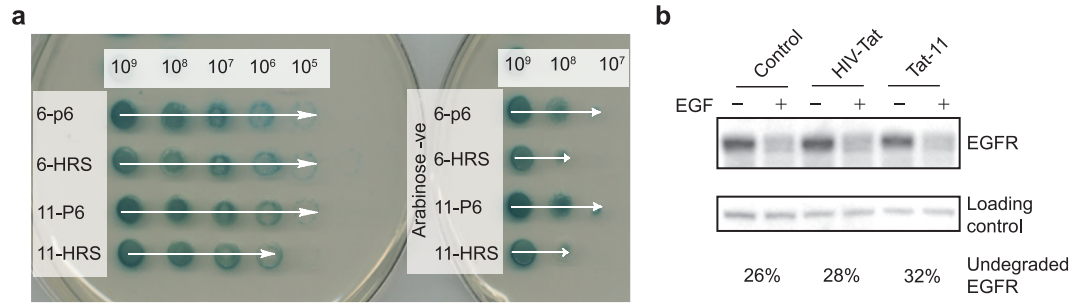

Figure 4. Effects of cyclic peptides on HRS—TSG101 interaction. a) Ranking of SICLOPPS produced cyclic peptide 6 and 11, in the p6—UEV and HRS—UEV strains by serial dilutions (2.5 mL of ~10^n cells/mL). The arabinose-deficient selection plate has significantly lower intracellular cyclic peptide levels (arabinose is the promoter for SICLOPPS). Comparing the two plates highlights the significant growth advantage conferred by the cyclic peptide inhibitors. b) Tat-tagged cyclic peptide 11 (10 μM) having no affect on EFGR degradation (a process controlled by HRS—TSG101) in HeLa cells, compared to the wild-type and the HIV—Tat sequence alone.

the PTAP mutant Gag VLP (Figure 3, panel c). These data indicate that cyclic peptide 11 specifically exerts its inhibitory effect by interfering with PTAP-dependent Gag budding.

Potentially, inhibitors of the TSG101—Gag interaction may also interfere with the TSG101—HRS interaction, affecting the cellular function of TSG101. In fact, we detected similar reduction (~50%) in the amount of HRS or Gag coprecipitated with TSG101 from 293T cell extracts in the presence of 10 μM Tat-tagged cyclic peptide 11, whereas the Tat sequence by itself (negative control) had no effect. In additional bacterial reverse two-hybrid experiments, SICLOPPS plasmids coding for cyclic peptides 6, 11, and 16 were introduced into an HRS—UEV selection strain. Disruption of the TSG101—Gag and TSG101—HRS interaction by each peptide was compared using drop spotting (Figure 4, panel a). The results also showed that both interactions were affected to the same extent by the selected cyclic peptides. We therefore set out to determine whether our genetically selected viral budding inhibitor also affects the ability of the TSG101—HRS complex to carry out its normal physiological function, assaying Tat-tagged cyclic peptide 11 for its effects on the ligand-induced degradation of epidermal growth factor receptor (EGRF) in HeLa cells (*18*). There was no detectable inhibition of EFGR degradation by Tat-tagged cyclic peptide 11 at 10 μM (Figure 4, panel b), the same concentration that reduced VLP release by 60%. This result is consistent with earlier evidence that the cellular level of TSG101 can be reduced sufficiently by RNAi to affect viral release without preventing cell growth (*12*).

The finding that interference with Tat-tagged cyclic peptides inhibits viral budding by targeting the TSG101 interaction without precluding the essential actions of TSG101 on receptor endocytosis and degradation suggests the therapeutic potential of small molecules that interfere with host protein functions recruited by viruses. As the viral budding process relies on host proteins, it may not be as readily circumvented by viral mutations as are virus functions targeted by current HIV countermeasures.

METHODS

Recombinant DNA Techniques. *Escherichia coli* cultures were maintained in LB broth. DNA manipulations were performed with *E. coli* DH5α-E (Invitrogen) cells. Plasmids were transformed into *E. coli* by heat shock or electroporation. Restriction and DNA-modifying enzymes were purchased from New England BioLabs. Oligonucleotides were purchased from Integrated DNA Technologies. PCR purification and gel extraction kits were purchased from Qiagen. DNA sequencing was performed at the Nucleic Acid Facility of the Pennsylvania State University.

Bacterial Reverse Two-Hybrid System. To construct the heterodimeric RTHS, the p6 region of HIV Gag was cloned as a fusion with a chimeric bacteriophage 434·P22 DNA binding repressor. The UEV domain of TSG101 was cloned as a fusion with the bacteriophage 434 repressor DNA binding domain. Both fusions were placed under control of an IPTG-inducible promoter. Antibiotics were provided at the following concentrations: ampicillin 100 μg mL^{-1}; chloroamphenicol 50 μg mL^{-1}; kanamycin 50 μg mL^{-1}; spectinomycin 50 μg mL^{-1}. For chromosomal markers, concentrations of antibiotics were reduced 2-fold. Mini-

mal media A supplemented with 0.5% glycerol and 1 mM $MgSO_4$ was used for all genetic selections.

Genetic Selection for Cyclic Peptides. SICLOPPS SGWXXXXX and SGWXXPXXPXX libraries (*23, 24*) were transformed into *E. coli* strains containing integrated reporter and repressor constructs. Transformants were washed with minimal media A and plated on minimal media A supplemented with 13 μM L-(+)-arabinose, 2.5 mM 3-amino-1,2,4-triazole, 25 μM kanamycin, and 30 μM IPTG. After incubation at 37 °C for 2−3 days, ~200 surviving colonies were picked and restreaked onto the same media with and without arabinose. Colonies that displayed an arabinose dependent growth advantage were tested for IPTG-dependent inhibition of growth (thus filtering out false positives potentially arising through damage to the expression of SICLOPPS or fusion proteins). Plasmids from strains that passed the above tests were retransformed into the original p6−UEV RTHS strain and rechecked for phenotype retention. The 10 remaining peptides were ranked for their activity by spotting serial dilutions of the corresponding cells onto selective media, allowing the conferred growth advantage to be compared at each dilution level. The variable insert regions on the SICLOPPS plasmids were PCR-amplified, and the DNA sequence of the active peptides was determined.

We examined the target specificity of the four most active selected cyclic peptides using a RTHS strain coding for the large and small subunits of murine ribonucleotide reductase (RR). The SICLOPPS plasmid of the p6−UEV selectants was transformed into the RR RTHS strain, and the activity of each cyclic peptide was ranked by drop spotting as before. The cyclic peptides that act *via* specific disruption of the p6−UEV interaction are expected to be inactive in the new RR RTHS strain. However, if the peptides confer arabinose-dependent growth advantage on the RTHS strain by a different mechanism, the selectants would also be active in the RR RTHS strain (which is identical to the p6−UEV RTHS strain except for the RR homodimer). One of the four selectants incurred a growth advantage (arabinose-dependent) on the RR RTHS strain, showing a lack of specificity toward inhibiting p6−UEV dimerization, and was therefore discarded.

Chemical Synthesis of Cyclic Peptides. Linear peptides were synthesized at the Hershey Macromolecular Core Facility of the Pennsylvania State University. Linear peptide 11 (WIYWNVSG, 10.0 mg, 9.8 μmol) was added to a solution of EDC (6 mg, 3 equiv, 29.4 μmol) and HOAt (8.5 mg, 6 equiv, 58.8 μmol) in DMF (15 mL). The mixture was agitated at RT for 24 h. The solvent was removed *in vacuo*, and the remaining residue was dissolved in 500 μL of DMF and added dropwise to 10 mL of diethyl ether. The resulting solid was separated by centrifugation and purified as outlined below (6.5 mg, 6.3 μmol, 64%). The procedure was repeated for peptides 6 (WTNWYGSG) and 16 (WTLLVYSG). Crude cyclic peptides were subjected to reverse-phase chromatography (Partisil C-18 Magnum 9 [length 50 cm, particle size 10 μM] ODS-3 columns, Whatman) on a Waters HPLC system by using a water/acetonitrile gradient with 0.1% trifluoroacetic acid. Mass analysis was performed on a Mariner mass spectrometer (PerSeptive Biosystems, Framingham, MA).

Mammalian Cell Culture and Transfection. 293T cells were maintained in DMEM supplemented with antibiotics and 10% FBS (Invitrogen). Plasmid DNA transfections were performed by using the FuGENE 6 reagent (Roche).

Antibodies, Western Blotting, and Immunoprecipitation (IP). Primary antibodies used include polyclonal anti-GFP antibody (Clontech), monoclonal anti-HRS antibody (Alexis Biochemical), and monoclonal anti-Flag antibody (Sigma-Aldrich). HRP-conjugated secondary antibodies were goat anti-mouse IgG and goat anti-rabbit IgG (Santa Cruz Biotechnology). Western blotting and Co-IP were performed using a procedure slightly modified from that previously described (*18*). Flag-tagged TSG101 was cotransfected with GFP-Gag into 293T cells for 48 h. Cells were lysed in the lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% NP40) with protease inhibitors (protease inhibitor tablet, Roche). Flag-TSG101 was captured using anti-Flag antibody conjugated agarose beads (Sigma-Aldrich). The immunoprecipitated complex was subjected to SDS−PAGE and Western blotting.

Gag VLP Assay. A GFP-tagged wild-type Gag expression construct (obtained from Dr. Marilyn Resh, Memorial Sloan-Kettering Cancer Center, New York (*30*)) or a GFP-tagged PTAP minus mutant Gag (PTAP changed to LIRL) construct (obtained from Dr. Wesley Sundquist, University of Utah (*12*)) was transfected into 293T cells. Forty-eight hours after the initiation of transfection, the culture supernatants were collected and filtered through a 0.45-μm-pore-size filter. Virus-like particles in the culture supernatants were isolated by ultracentrifugation through a cushion of 20% sucrose at 45,000 rpm for 90 min at 4 °C using a Beckman SW50.1Ti rotor. The viral particles in the pellets were resuspended in SDS sample loading buffer and subjected to SDS−PAGE and Western blotting.

Ligand-Induced EGFR Downregulation Assay. 293T cells were seeded in six-well plates at 5×10^5 per well and transfected with 50 ng of pCDNA3-EGFR (obtained from Dr. Hamid Band, Northwestern University (*28*)). Forty-eight hours after the initiation of transfection, the cells were starved in serum-free medium for 1 h and were either mock-treated as controls or incubated with EGF (Sigma-Aldrich) at a concentration of 150 ng mL^{-1} in 37 °C for 90 min. Cells were then washed with cold PBS and immediately lysed. Lysates were subjected to SDS−PAGE and Western blotting using rabbit anti-EGFR antibody (Santa Cruz Biotechnology).

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