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Tsg101 and the Vacuolar Protein Sorting Pathway Are Essential for HIV-1 Budding

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Summary

Like other enveloped viruses, HIV-1 uses cellular machinery to bud from infected cells. We now show that Tsg101 protein, which functions in vacuolar protein sorting (Vps), is required for HIV-1 budding. The UEV domain of Tsg101 binds to an essential tetrapeptide (PTAP) motif within the p6 domain of the structural Gag protein and also to ubiquitin. Depletion of cellular Tsg101 by small interfering RNA arrests HIV-1 budding at a late stage, and budding is rescued by reintroduction of Tsg101. Dominant negative mutant Vps4 proteins that inhibit vacuolar protein sorting also arrest HIV-1 and MLV budding. These observations suggest that retroviruses bud by appropriating cellular machinery normally used in the Vps pathway to form multivesicular bodies.

Introduction

HIV-1 assembly is driven by the viral Gag protein, which is actively trafficked to the plasma membrane where it associates into enveloped, spherical particles that bud from the cell (reviewed in Freed, 1998). During viral assembly, Gag is processed by the viral protease at a series of sites to produce four new structural proteins that perform essential functions in the mature, infectious virion (denoted MA, CA, NC, and p6; Figure 1A). Proteolytic processing is not required for particle production, however, as HIV-1 Gag can assemble and bud in the absence of any other viral proteins.

The budding of an enveloped virus can be viewed as a fission event in which the continuous cell membrane is broken and resealed to create discrete viral and cellular membranes. Like other enveloped viruses, HIV-1 does not encode its own membrane fission machinery, and presumably therefore must recruit and reprogram cellular proteins to assist in the budding process. A potential docking site for such a cellular factor(s) has been mapped to a conserved P(T/S)AP motif located in the p6 domain of HIV-1 Gag. Point mutations within this "PTAP" motif arrest viral release at a very late stage (Göttlinger et al., 1991; Huang et al., 1995), and it has therefore been termed a "late domain" (Wills and Craven, 1991). Virus assembly appears to initiate normally in the late domain mutants, but the continuous membrane that connects the budding particles to the cell (or to other budding particles) is not severed, resulting in abnormal or severely attenuated virus release.

The recruitment of cellular machinery to facilitate virus budding seems to be a general phenomenon, and distinct late domains have been identified in the structural proteins of many enveloped viruses (Vogt, 2000). Two well-characterized late domains are the "PY" motif (consensus sequence: PPXY; X = any amino acid) found in membrane-associated proteins from filo-, orbi-, rhabdo- and oncoretroviruses (Craven et al., 1999; Harty et al., 1999, 2000; Jayakar et al., 2000), and the "YL" motif (YXXL) found in the Gag protein of *e*quine *i*nfectious *a*nemia *v*irus (EIAV) (Puffer et al., 1997, 1998). The various late domains can still function when moved to different positions within retroviral Gag proteins, supporting the idea that they are docking sites for cellular factors rather than structural elements (Parent et al., 1995; Yuan et al., 2000). Moreover, the different late domains can function interchangeably, and multiple late domains are often found in close proximity within viral coat proteins, suggesting that they may act synergistically (Parent et al., 1995; Strack et al., 2000; Yuan et al., 2000).

Ubiquitin (Ub) also plays an essential, albeit poorly understood, role in retroviral budding (Patnaik et al., 2000; Schubert et al., 2000b; Strack et al., 2000; Vogt, 2000). For example, retrovirus budding can be blocked at a late stage by depleting cellular pools of free ubiquitin with proteasome inhibitors. It is not yet certain, however, whether the functionally relevant substrate for ubiquitination is Gag itself or a cellular factor. HIV-1 p6 and other retroviral Gag proteins are monoubiquitinated at low levels (Ott et al., 2000), and there is a general correlation between Gag ubiquitination and virus release (Schubert et al., 2000b; Strack et al., 2000). Moreover, the block imposed by proteasome inhibitors can be at least partially alleviated by covalently fusing Ub to the C-terminal end of *R*ous *s*arcoma *v*irus (RSV) Gag (Patnaik et al., 2000). However, virus release and replication are not affected by mutation of the two HIV-1 p6 lysine residues (Lys-27 and Lys-33) that are the major sites for Gag ubiquitination (Ott et al., 2000). The functional role of Gag ubiquitination therefore remains uncertain. In principle, Gag ubiquitination could facilitate budding either by targeting defective Gag molecules for proteolytic degradation and thereby preventing them from interfering with viral budding (Schubert et al., 2000a), or by creating docking sites for cellular factors that actively participate in viral budding.

To understand how HIV-1 and other enveloped viruses bud, it is essential to define the participating cellular factors. We now report that human *t*umor *s*usceptibility *g*ene 101 (Tsg101) is required for the release of infectious HIV-1 and suggest that the protein facilitates budding by linking the p6 late domain to vacuolar protein sorting machinery.

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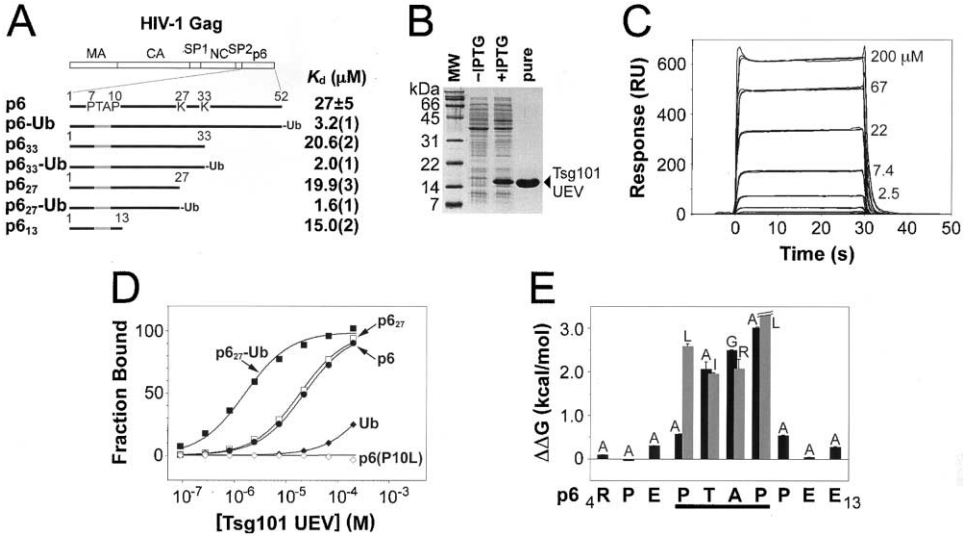

Figure 1. Human Tsg101 Binds HIV-1 p6

(A) HIV-1 Gag domain structure and dissociation constants for pure Tsg101 UEV protein binding to immobilized HIV-1 GST-p6 and GST-p6-Ub proteins. The standard deviation in the dissociation constant for the full-length, wild-type p6 protein was derived from 16 independent measurements; estimated errors in the final significant digits of the other dissociation constants (parentheses) were derived from statistical fits of individual isotherms to 1:1 binding models.

(B) SDS-PAGE analysis showing induced expression in *E. coli* and fully purified Tsg101 UEV.

(C) Surface plasmon resonance biosensor analysis of the p6/Tsg 101 interaction. Tsg101 UEV was injected in triplicate at concentrations of 0, 0.9, 0.27, 0.82, 2.5, 7.4, 22, 67, and 200 μM over GST-p6 captured on an anti-GST surface. Data were double referenced to correct for bulk refractive index changes and any nonspecific binding (Myszka, 1999).

(D) Representative binding isotherms for Tsg101 UEV interacting with a series of different protein surfaces.

(E) Epitope mapping of the Tsg101 UEV binding site on HIV-1 p6. The graph shows the changes in free energy of Tsg101 UEV binding (ΔΔG) caused by single alanine substitutions in HIV-1 p6 residues 4–13 (dark bars) or by the P7L, T8I, A9R, and P10L mutations (light bars). Positive values indicate that the mutation reduces binding affinity, and error bars show estimated standard deviations in single binding isotherms. Broken lines above the P10L mutant denote a lack of detectable Tsg101 UEV binding (see also Figure 1D).

Results

Human Tsg101 Binds Specifically to the PTAP Motif of HIV-1 p6

Carter and coworkers have recently reported using a yeast two-hybrid screen to identify Tsg101 as a p6 binding protein (VerPlank et al., 2001). We independently used a similar strategy to screen for proteins that could bind to the PTAP late domain within HIV-1 p6 and obtained similar results. Briefly, HIV-$1_{NYU/BR5}$ p6 was used as bait to screen a human spleen cDNA prey library for potential binding partners. Genes encoding nearly full-length Tsg101 were isolated twice, and were the only genes detected and confirmed in these screens. In subsequent experiments, we showed that full-length Tsg-101 bound wild-type p6 in directed two-hybrid liquid culture assays, resulting in high levels of β-galactosidase activity (>300-fold over background; not shown). Three different p6 point mutants (P7L, A9R, and P10L) were used to test whether the Tsg101 binding interaction required the PTAP motif within HIV-1 p6, and all three reduced β-galactosidase activity to background levels. Each of these point mutations also arrests HIV-1 budding at a late stage (Huang et al., 1995). Hence, Tsg101 was judged an attractive candidate for the cellular factor that binds the PTAP late domain of HIV-1 p6 and facilitates viral budding.

Directed yeast two-hybrid experiments were further used to map the primary p6 binding site to the N-terminal half of Tsg101 (residues 1–207, data not shown). Comparative sequence analyses have suggested that this region of Tsg101 contains a domain that belongs to the *u*biquitin enzyme 2 *v*ariant (UEV) protein family (Koonin and Abagyan, 1997; Ponting et al., 1997; VanDemark et al., 2001). UEV proteins are homologous to the E2 class of ubiquitin conjugating enzymes, but lack the active site cysteine residue required to make a transient thioester bond during ubiquitin transfer, and are presumably not enzymatically active. A construct spanning Tsg101 residues 1–145 was used to characterize the interaction between HIV-1_{NL4-3} p6 and the Tsg101 UEV domain in vitro. Recombinant Tsg101 UEV was expressed in *E. coli*, purified to homogeneity, and tested for binding to immobilized GST-p6 fusion proteins using a surface plasmon resonance biosensor (Figure 1). Tsg101 UEV exhibited concentration-dependent p6 binding, and equilibrium responses fit a simple 1:1 binding model with an equilibrium dissociation constant ($K_d^{20°C}$) of 27 ± 5 μM. As summarized in Figure 1A, a series of constructs spanning p6 residues 1–13, 1–27, 1–33, and 1–52 (full-length p6) all bound Tsg101 UEV with similar affinities. Thus, the primary Tsg101 binding site is located within the first 13 residues of p6.

Site-directed mutagenesis of p6 was then used to map the Tsg101 binding site more precisely. Residues 4–13 within full-length p6 were substituted individually with alanine (Figure 1E, dark bars) or with residues known to block virus release (light bars). Mutations in the

four central PTAP residues significantly reduced Tsg101 binding (>2 kcal/mol loss of binding free energy). Mutations at three flanking sites (E6A, P11A, and E13A) moderately reduced the binding affinity (0.3–0.5 kcal/mol), indicating that these residues also contributed to Tsg101 UEV binding. Alanine substitutions at three other sites (R4A, P5A, and E12A) had no significant effect on Tsg101 UEV binding. These experiments demonstrate that the Tsg101 UEV domain binds directly and specifically to HIV-1 p6 and that the p6 PTAP motif is the energetically dominant binding epitope. Our data are in excellent agreement with the study of VerPlank et al., who also showed that Tsg101 binding was PTAP-specific (VerPlank et al., 2001).

Ubiquitin Modification of HIV-1 p6 Enhances Tsg101 Binding

We reasoned that ubiquitination of HIV-1 p6 might enhance Tsg101 UEV binding, because other UEV proteins can bind ubiquitin (VanDemark et al., 2001) and because ubiquitin is required for HIV-1 budding (Schubert et al., 2000b; Strack et al., 2000). The enzymes that conjugate Ub onto HIV-1 Gag are not yet known, so models for p6-Ub conjugates were created by fusing the Ub protein in frame to the C termini of full-length p6, $p6_{33}$, and $p6_{27}$ constructs (Figure 1A). Analogous approaches have been employed by others to rescue Ub-dependent defects in endocytosis (Shih et al., 2000) and RSV budding (Patnaik et al., 2000). In all three p6-Ub constructs, the presence of ubiquitin *increased* the affinity of Tsg101 UEV binding approximately 10-fold (ave. $K_d = 2.3\ \mu M$; Figures 1A and 1D). Tsg101 UEV even bound weakly to Ub alone (Figure 1D, est. $K_d = 510 \pm 35\ \mu M$). These experiments demonstrate that Tsg101 UEV, though lacking enzymatic activity, has retained Ub binding activity and can bind cooperatively to ubiquitin and to the HIV-1 p6 late domain. We envision that Tsg101 could bind ubiquitinated Gag molecules even more tightly in vivo if the UEV domain senses the native Ub isopeptide geometry or if oligomerization of full-length Tsg101 increases its avidity for the assembling Gag lattice.

Tsg101 Is Required for Efficient Release of HIV-1 from 293T Cells

It was important to develop systems to test the requirement for cellular Tsg101 in HIV-1 budding. Others have shown that the HIV-1 p6 late domain can mediate virus release from human embryonic kidney 293T cells (Yuan et al., 2000), and we confirmed that a series of mutations in the p6 PTAP sequence that block HIV-1 release and replication in other cell lines also blocked release of infectious HIV-1 particles in 293T cells (data not shown). We next tested whether we could deplete Tsg101 from 293T cells using small interfering RNAs (siRNA) (Elbashir et al., 2001). The system was optimized until it became possible to deplete endogenous Tsg101 to nearly undetectable levels (Figure 2A, lanes 2, 8, and 10). Briefly, 293T cells were transfected twice at 24 hr intervals with a small interfering RNA duplex homologous to nucleotides 413–433 of the *Tsg101* coding sequence (denoted siRNA). Tsg101 levels were analyzed after an additional 24 hr by Western blotting of cytoplasmic extracts from the bulk culture. Under these conditions, traces of

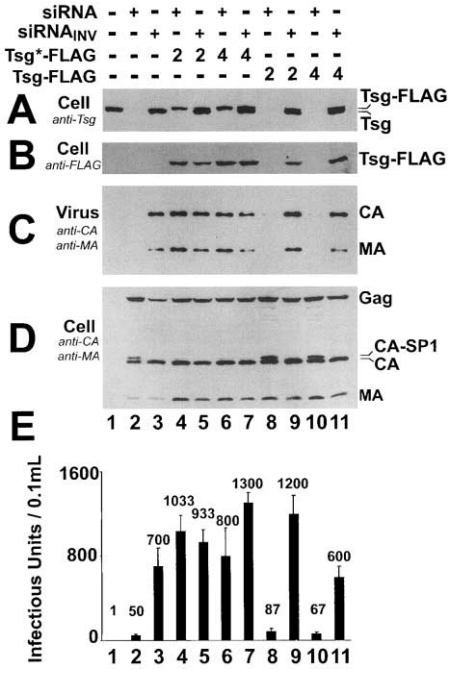

Figure 2. Tsg101 Is Required for Release of HIV-1 from 293T Cells

(A) Anti-Tsg101 Western blot showing depletion of Tsg101 protein from 293T cells by siRNA and restoration of Tsg101 protein using siRNA-resistant expression constructs. Cells were cotransfected twice (2 ml cultures, 6-well plates) with plasmid DNA and with 50 nM siRNA or siRNA$_{INV}$ at 24 hr intervals (except mock, lane 1, which received no RNA or DNA). The first siRNA/siRNA$_{INV}$ cotransfection was with 2 μg or 4 μg of a Tsg-FLAG (siRNA-sensitive) or Tsg*-FLAG (siRNA-resistant) expression construct. The second siRNA/siRNA$_{INV}$ cotransfection was with 0.5 μg HIV-1 R9 DNA. In each case, total transfected DNA was normalized to 4.5 μg with pIRES2-EGFP. Cytoplasms and supernatants were analyzed 48 hr after the first transfection.

(B) Anti-FLAG Western blot showing that the Tsg101*-FLAG construct is resistant to siRNA. Samples were prepared in (A).

(C) Anti-MA and anti-CA Western blot showing that depletion of Tsg101 inhibits virus release. From cells transfected in (A), viral particles were pelleted from the supernatant of the transfected cells. Virus release in untreated cells was similar to that in the siRNA$_{INV}$-treated cells (not shown).

(D) Anti-MA and anti-CA Western blot showing that cytoplasmic levels of HIV-1 Gag, CA, and MA proteins are not affected by Tsg101 depletion. Note that levels of MA and CA in cellular membrane fractions increased significantly when virus release was blocked (not shown).

(E) Viral replication assays showing that release of infectious virus is inhibited when Tsg101 is depleted and rescued when Tsg101 is restored. Titers of infectious HIV-1 particles released into the supernatant (shown) were quantitated in single cycle MAGIC assays.

Tsg101 protein could only be detected in highly overexposed Western blots, whereas levels of control proteins were unaffected (not shown). Nuclear and membrane fractions were also depleted of Tsg101 (not shown). A heterologous control RNA duplex of inverted sequence (denoted siRNA$_{INV}$) did not affect cellular Tsg101 levels, demonstrating the specificity of siRNA. Although others have reported that altering Tsg101 levels can affect cell proliferation (Zhong et al., 1998), we observed only a very slight growth reduction in Tsg101-depleted cells over the 72 hr time course.

Tsg101 protein was reintroduced into Tsg101-depleted cells by cotransfecting a plasmid encoding FLAG-tagged Tsg101 protein with 7 silent mutations at the siRNA target site that rendered the gene resistant to RNA interference (denoted Tsg*-FLAG). As shown in Figures 2A and 2B, this "resistant" Tsg*-FLAG protein was not depleted by siRNA (lanes 4 and 6). In contrast, endogenous Tsg101 and exogenous Tsg-FLAG expressed from wt genes were always efficiently depleted by siRNA treatment.

To test whether Tsg101 was required for HIV-1 budding, Tsg101-depleted cells were cotransfected with a proviral HIV-1 R9 expression construct during the second siRNA transfection. Depletion of Tsg101 very significantly reduced the release of virion-associated MA and CA proteins as analyzed in Western blots, and also reduced viral infectivity in single cycle MAGIC infectivity assays (Figures 2C and 2E, lanes 2, 8, and 10). Viral titers were reduced 10- to 50-fold in multiple repetitions of this experiment. Both virus release and infectivity were restored to normal levels when the Tsg*-FLAG protein was reintroduced (lanes 4 and 6), formally demonstrating that Tsg101 is required for the release of infectious HIV-1 from 293T cells. Reductions in virus release in the Tsg101-depleted cells did not reflect general defects in HIV-1 Gag protein synthesis, stability, or processing (Figure 2D). However, Tsg101 depletion reproducibly increased accumulation of the CA-SP1 Gag processing intermediate in the cytoplasm, consistent with a defect in the final stages of particle assembly (Gottlinger et al., 1991).

We also tested the effect of Tsg101 depletion on the release of murine leukemia virus (MLV), which uses the alternative "PY" late domain pathway (Yuan et al., 1999). As expected, mutating the MLV Gag p12 PPPY motif (to AAAA) blocked particle release and infectivity (Figure 3). However, depletion of Tsg101 had only a minor effect, if any, on MLV release and infectivity. These experiments demonstrate that Tsg101-depleted 293T cells can still support replication of retroviruses other than HIV-1, provided they are not released via the PTAP-dependent pathway.

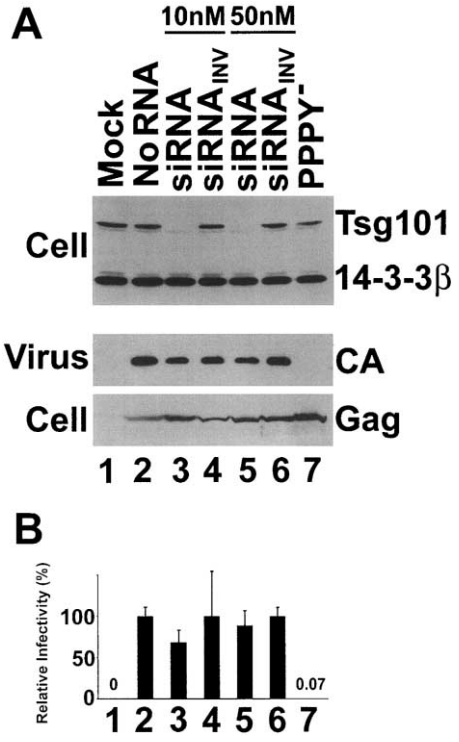

Figure 3. Depletion of Tsg101 Does Not Affect MLV Release or Infectivity

(A) Depletion of Tsg101 does not significantly inhibit MLV release from 293T cells.

Upper panel: Anti-Tsg101 and anti-14-3-3β Western blot showing specific depletion of Tsg101 from 293T cells by siRNA. Depletion experiments were performed as described in Figure 2A, except that the second siRNA cotransfection was with 1.5 μg pCLeco and 0.25 μg pCLlacZ MLV vector DNA. Levels of 14-3-3β protein are shown as loading controls, and a pCLeco vector expressing PPPY$^-$ mutant Gag is shown as a positive control for arrested budding (lane 7).

Middle panel: Anti-MLV CA Western blot showing that virus release is not significantly reduced by Tsg101 depletion.

Lower panel: Anti-CA Western blot showing cytoplasmic levels of MLV Gag.

(B) Depletion of Tsg101 does not significantly inhibit MLV infectivity. Infectious MLV particles released into the supernatant were quantitated in single cycle infectivity assays and normalized to the appropriate controls. In repetitions of these experiments, siRNA treatment reduced particle release and infectivity slightly (up to 2-fold) relative to the siRNA$_{INV}$ control.

A Functional Vps Pathway Is Required for HIV-1 Release from 293T Cells

Tsg101 functions in the *v*acuolar *p*rotein *s*orting (Vps) pathway, in which membrane-associated proteins are sorted through a series of endosomal compartments for eventual degradation in the lysosome (Lemmon and Traub, 2000). To test whether Tsg101 might link viral budding to the cellular Vps pathway, we examined the effects of other "functional knockouts" of the Tsg101/Vps pathway on HIV-1 budding. Although Tsg101 is predominantly cytosolic at steady state (Xie et al., 1998; Zhong et al., 1998), the protein appears to cycle dynamically through the endosomal network. Another protein in the Vps pathway, Vps4, functions in Tsg101 cycling and endosomal trafficking (Babst et al., 1998; Bishop and Woodman, 2000, 2001). Vps4 is an ATPase of the AAA protein family that appears to utilize the energy derived from ATP hydrolysis to disassemble endosome-associated Vps complexes and thereby allow multiple rounds of sorting. Overexpressing ATPase-defective GFP-Vps4 fusion proteins induces formation of enlarged endosomes (called "class E" compartments in yeast) that are defective in the sorting and recycling of endocytosed substrates (Bishop and Woodman, 2000). The Vps4 mutants also prevent normal Tsg101 trafficking because the protein is trapped on the surface of these aberrant endosomes (Bishop and Woodman, 2001).

To test the requirement for Tsg101/Vps function in HIV-1 particle release, vectors expressing ATPase-defective GFP-Vps4 proteins were cotransfected together with the proviral HIV-1 R9 expression vector. Two different dominant negative mutations were used: K173Q, which blocks ATP binding (Vps4$_{K173Q}$), and E228Q, which blocks ATP hydrolysis (Vps4$_{E228Q}$). Protein levels and viral infectivity were again analyzed by Western blotting and in MAGIC assays. As shown in Figures

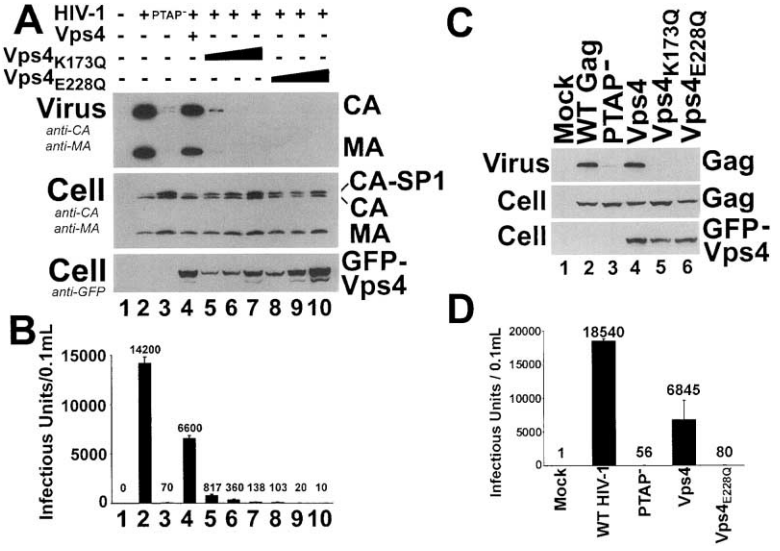

Figure 4. Mutant Vps4 Proteins Dominantly Inhibit HIV-1 Release and Infectivity

(A) Mutant Vps4 proteins dominantly inhibit HIV-1 release. 293T cells were transfected with 0.5 μg R9 plasmid encoding wt HIV-1 (lanes 2 and 4–10) or a p6 PTAP$^-$ mutant HIV-1 (lane 3). Cells were cotransfected with pEGFP (1 μg, lane 2), GFP-Vps4 wt (1 μg, lane 4), GFP-Vps4$_{K173Q}$ (0.25, 0.5, or 1 μg; lanes 5–7), or GFP-Vps4$_{E228Q}$ (0.25, 0.5, or 1 μg; lanes 8–10). Total DNA was normalized to 1.5 μg with pEGFP. Pelleted virus (above) and cytoplasmic extracts (labeled Cell, below) were harvested after 24 hr, and levels of expressed CA, MA, and GFP-Vps4 proteins analyzed by Western blotting.

(B) Mutant Vps4 proteins dominantly inhibit HIV-1 infectivity. Levels of infectious HIV-1 particles released into the supernatant were quantitated using single cycle MAGIC assays. Samples were from (A).

(C) Mutant Vps4 proteins dominantly inhibit release of Gag-GFP. 293T cells were transfected with 1 μg vector expressing wt Gag-GFP (lanes 2 and 4–6) or a p6 PTAP$^-$ mutant Gag-GFP (lane 3). Samples were cotransfected with 1 μg vector expressing GFP only (lane 2), wt GFP-Vps4 (lane 4), GFP-Vps4$_{K173Q}$ (lane 5), or GFP-Vps4$_{E228Q}$ (lane 6). Pelleted virus-like particles (above) and cytoplasmic extracts (below) were harvested after 24 hr, and Gag and GFP-Vps4 expression were analyzed by Western blotting with anti-MA, anti-CA (top two panels), and anti-GFP antibodies (bottom panel).

(D) PTAP$^-$ mutation and dominant negative Vps4 proteins inhibit release of infectious HIV-1 from MT4 T cells. Cells were transfected (24-well plates) with 1 μg HIV-1 R9 DNA and 1 μg Vps4 DNA or 1 μg control DNA (pEGFP). Levels of infectious HIV-1 particles released into the supernatant were quantitated using single cycle MAGIC assays.

4A and 4B, coexpression of the mutant GFP-Vps4 proteins inhibited particle production in a dose-dependent fashion, with release of infectious particles reduced more than 1000-fold at the highest levels of Vps4$_{E228Q}$ tested. Cotransfection with the wild-type GFP-Vps4 construct reduced particle production and infectivity only slightly (~2- to 3-fold). This reduction was reproducible, however, and may reflect stoichiometric imbalances in the Vps pathway arising from Vps4 overexpression. Expression levels for all of the GFP-Vps4 proteins paralleled the levels of transfected DNA (as detected by anti-GFP Western, Figure 4A, bottom), and none of the Vps4 proteins altered Gag protein synthesis, stability, or cell viability significantly (Figure 4A, middle). Other controls for this experiment included: (1) mock transfections (lane 1), (2) cotransfection of R9 with a vector expressing GFP only (no effect on budding; lane 2), and (3) transfection of an R9 vector encoding a PTAP to LIRL mutant HIV-1 provirus (arrested budding; lane 3).

As the Vps4 mutants interfered with endosomal trafficking, we considered the possibility that particle release might have been inhibited by altered trafficking, activation, or assembly of viral proteins other than Gag (e.g., protease (PR), envelope (Env), or viral protein U). This was not the case, however, because the mutant GFP-Vps4 proteins also blocked release of viral constructs missing PR or Env (data not shown) and even blocked release of Gag-GFP alone (Figure 4C, lanes 5 and 6). As expected, Gag-GFP was released efficiently in the absence of the dominant negative Vps constructs, but was blocked by mutation of the Gag p6 PTAP sequence (lane 3). These experiments demonstrate that the Vps4-induced block to HIV-1 release does not require any other viral proteins, consistent with the idea that the p6 domain of the assembling Gag particle recruits components of the Tsg101/Vps pathway to assist in budding.

A Functional Vps Pathway Is Required for Release of Infectious HIV-1 from Human T Cell Lines

We also tested whether an intact PTAP motif and a functional Vps pathway were required for release of infectious HIV-1 particles from T cells, which are HIV-1 hosts in vivo. Although the transfection efficiencies of T cell lines are low, viral titers produced from MT4 cells 72 hr posttransfection were sufficiently high to quantitate using the MAGIC assay. Mutation of HIV-1 p6 PTAP reduced viral titers >300-fold (Figure 4D) and blocked spreading infections in MT4 cultures (growth curves not shown). Thus, HIV-1 also requires p6 PTAP to replicate in this nonadherent T cell line. Inhibition of the Vps pathway by overexpression of the dominant negative Vps4$_{E228Q}$ mutant also severely reduced infectious HIV-1 titers (>200-fold), whereas overexpression of the wt Vps4 protein had only modest effects (~3-fold reduction). Similar results were obtained in the CEMss T cell line (data not shown). Thus, we conclude that HIV-1 requires both a PTAP motif and a functional Vps pathway to replicate in multiple cell types, including T cells.

A Functional Vps Pathway Is Required for Release of Infectious MLV from 293T Cells

The effects of mutant GFP-Vps4 protein overexpression on MLV particle release and infectivity in 293T cells were also tested (Figure 5). In this case, virion-associated CA release was *blocked*, with a concomitant increase in cytoplasmic levels of the unprocessed Gag protein (lanes 5 and 6). Similarly, infectivity was dramatically reduced (>800-fold for Vps4$_{E228Q}$). Thus, the block to viral release imposed by the Vps4 mutants is more general

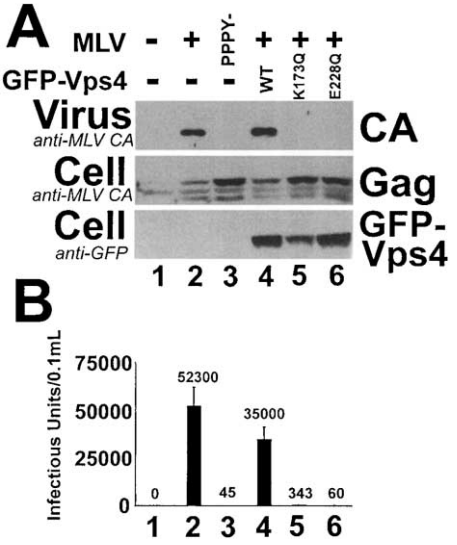

Figure 5. Mutant Vps4 Proteins Dominantly Inhibit MLV Release and Infectivity

(A) 293T cells were cotransfected with 1.5 μg pCLeco and 0.25 μg pCLlacZ MLV vectors encoding wt (lanes 2 and 4–6) or PPPY$^-$ mutant MLV Gag proteins (lane 3). Cotransfections were with 1 μg of the following vectors: pEGFP expressing GFP only (lanes 2, 3), wt GFP-Vps4 (lane 4), GFP-Vps4$_{K173Q}$ (lane 5), or GFP-Vps4$_{E228Q}$ (lane 6). Pelleted virus (upper panel) and cytoplasmic extracts (lower panels) were harvested after 24 hr, and levels of expressed MLV CA, Gag, and GFP-Vps4 proteins were analyzed by Western blotting with anti-MLV CA and anti-GFP antibodies as indictated.

(B) Mutant Vps4 proteins dominantly inhibit MLV replication. Samples were prepared as described in Figure 5A and MLV vector titers were quantitated as described in Experimental Procedures.

than that imposed by depleting Tsg101, which appears to be HIV-specific. These observations indicate that late endosomal trafficking, which is inhibited by the dominant negative Vps4 proteins, is required for virus release through *both* the PTAP and PPPY motifs.

Tsg101 Depletion and Vps4 Mutations Inhibit Late Stages of Virus Budding

Thin section electron microscopy was used to characterize the defects in viral release from Tsg101-depleted and Vps4-inhibited cells. In both experiments, HIV-1 budding arrested with "late domain" phenotypes that were very similar to those observed for control PTAP$^-$ mutant viruses (Figure 6A). In each case, isolated immature particles remained connected to the plasma membrane via membrane stalks, and budding particles also often formed "clusters" of interconnected virions. Although isolated budding HIV-1 particles were occasionally observed in control cells, these "late domain" mutant phenotypes were rarely seen. The dominant negative Vps4 proteins also arrested MLV particle release at a late stage, with a phenotype that was very similar to that observed for control PPPY$^-$ mutant viruses (Figure 6B). Thus, HIV-1 and MLV Gag proteins localized to the plasma membrane of both Tsg101-depleted and Vps4-inhibited cells, and appeared to initiate assembly normally. However, the viral particles failed to complete membrane fission, implying that Tsg101 (for HIV-1) and other components of the Vps pathway (for both HIV-1 and MLV) participate in this final stage of viral particle release.

Discussion

Our experiments demonstrate a requirement for Tsg101 in HIV-1 budding. In summary, Tsg101 UEV binds specifically to the PTAP motif within p6 (VerPlank et al. (2001) and this work), and there is an excellent correlation between mutations that inhibit Tsg101 binding and those that inhibit virus release (Huang et al., 1995). Depleting Tsg101 or inhibiting endosomal trafficking also arrests HIV-1 release at a late stage, indicating that Tsg101 and the Vps pathway perform essential functions in viral budding. Thus, these proteins represent potentially attractive new targets for therapeutic intervention.

The studies described herein also demonstrate the power of using the siRNA method developed by Tuschl and coworkers to test the functional requirement for a protein in a biological process (Elbashir et al., 2001). We have extended the utility of this technique by showing that exogenous, siRNA-resistant protein expression constructs can be used in functional rescue experiments. In principle, this same approach can also be used to test the functional phenotypes of site-directed mutant proteins in mammalian cells. More generally, these siRNA techniques are complementary to yeast two-hybrid and surface plasmon resonance experiments, and can be combined to reveal potential new binding partners for a target protein, characterize their interactions, and analyze their biological functions.

Protein Binding Motifs in Tsg101 UEV

We have shown that Tsg101 UEV is a multifunctional domain that can simultaneously bind both ubiquitin and the p6 PTAP motif, suggesting that Tsg101 may detect, or possibly participate in, the Ub transfer event required for HIV-1 budding. One attractive model is that ubiquitination of HIV-1 Gag during viral assembly creates high affinity binding sites that recruit Tsg101 to assist in the final stages of budding. Alternatively, Tsg101 could bind p6 and recruit a ubiquitinated cellular factor to the site of viral assembly. A recent crystallographic analysis of the yeast Mms2/Ubc13 complex has confirmed the overall similarity of the UEV (Mms2) and E2 (Ubc13) folds (VanDemark et al., 2001). In that complex, one face of Mms2 binds Ubc13, creating an active heterodimeric E2 enzyme. A second Mms2 surface then helps bind protein-conjugated Ub molecules and position their Lys-63 side chains to accept the C-terminal donor ends of incoming Ub molecules in the growing poly-Ub chain. We have recently determined the three-dimensional structure of Tsg101 UEV and find that it also exhibits an E2-like fold, but that the final two helices in the canonical E2 fold are replaced by the PTAP binding site (O.W.P. et al., unpublished data). Thus, both Mms2 and Tsg101 UEV couple Ub binding with other protein recognition motifs to facilitate the assembly of multiprotein complexes, and this may be a general function of UEV proteins.

Tsg101 and the Vps Pathway

Tsg101 was initially discovered in a screen for potential tumor suppressors (Li and Cohen, 1996), and the protein

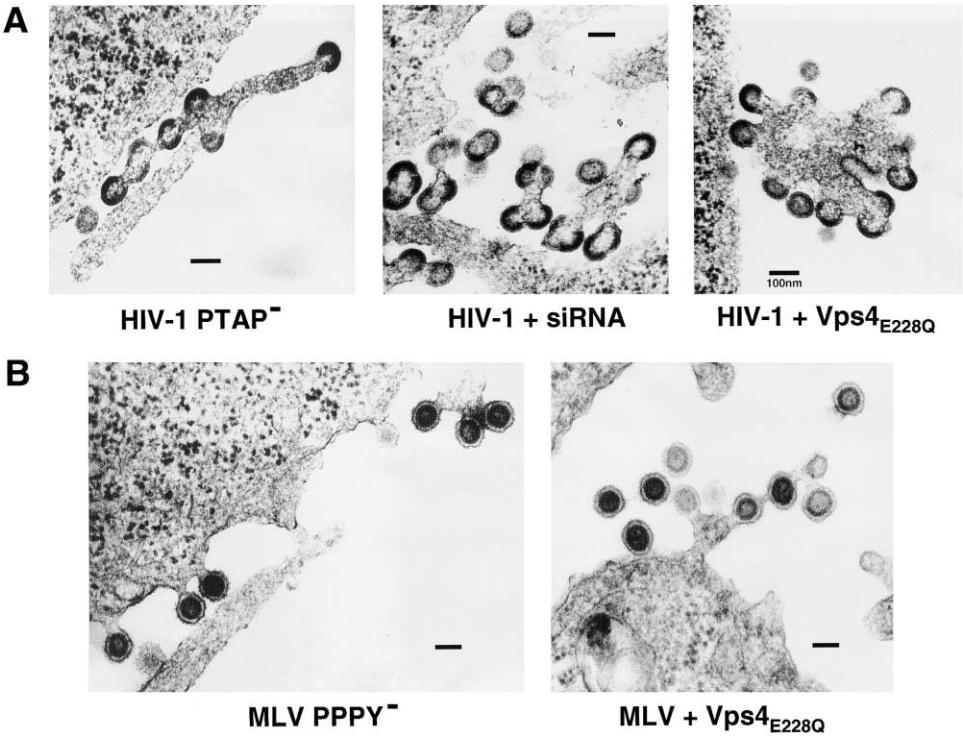

Figure 6. Tsg101 Depletion and Vps4 Mutants Block Virus Budding at a Late Stage

(A) Representative thin section electron micrographs showing the effects on HIV-1 budding of mutating the p6 PTAP sequence (left), depleting cellular Tsg101 (center), or overexpressing the dominant negative $Vps4_{E228Q}$ protein (right). In each case, virus budding arrested at a late stage, with immature particles remaining connected to the plasma membrane via membrane stalks or to other budding particles to form "clusters" of interconnected particles. A subset of the cells transfected with the siRNA and mutant Vps4 constructs exhibited what appeared to be Class E compartments in the plane of section, and a full description will be published elsewhere. Scale bars are all 100 nm.

(B) Representative thin section electron micrographs showing the effects on MLV budding of mutating the p12 PPPY motif (left) or overexpressing the dominant negative $Vps4_{E228Q}$ protein (right). These MLV late domain phenotypes are similar to those described above for HIV-1.

appears to perform multiple functions, including downregulating p53 via the MDM2/p53 pathway (Li et al., 2001; Ruland et al., 2001). Tsg101 also plays a central role in vacuolar protein sorting. The Vps pathway sorts membrane-bound proteins for eventual degradation in the lysosome (vacuole in yeast) (Lemmon and Traub (2000) and references therein). Two alternative entrées into the Vps pathway are via vesicular trafficking from the Golgi (e.g., in destroying misfolded membrane proteins) or via endocytosis from the plasma membrane (e.g., in downregulating surface receptors). Vesicles carrying proteins from either source can enter the Vps pathway by fusing with endosomes. As these endosomes mature, their cargoes are sorted for lysosomal degradation via the formation of structures called multivesicular bodies (MVB). MVB are created when surface patches on late endosomes bud into the lumen, forming small (~50–100 nm) vesicles. A maturing MVB can contain tens or even hundreds of these vesicles. The MVB then fuses with the lysosome, releasing the vesicles for degradation in this hydrolytic organelle.

A number of studies indicate that Tsg101 plays a central role in the Vps pathway. Deletion of the yeast Tsg101 ortholog, Vps23 (Stp22): (1) gives rise to a class E Vps phenotype, (2) blocks vacuolar protein sorting from the Golgi, and (3) inhibits surface receptor downregulation (Babst et al., 2000; Li et al., 1999). Very recent work from the Emr laboratory has demonstrated that Vps23 binds Vps28 and Vps37 to form the "ESCRT-1" complex, which selects cargo for incorporation into the lumen of the MVB (Katzmann et al., 2001). Importantly, both Vps23 and ESCRT-1 recognize mono- and diubiquitinated substrates, and lumenal MVB targeting in vivo requires substrate ubiquitination. These observations are in good accord with previous studies demonstrating functional roles for Ub conjugation and hydrolysis in targeting proteins into the yeast Vps pathway (Amerik et al., 2000; Dunn and Hicke, 2001; Dupre and Haguenauer-Tsapis, 2001; Galan et al., 2001; Losko et al., 2001; Wang et al., 2001). Although the mammalian Vps pathway is not yet as well characterized, Tsg101 and Vps23 appear to perform similar roles in vacuolar protein sorting (Babst et al., 2000; Bishop and Woodman, 2001). At this stage, however, only Tsg101 has been shown to bind to PTAP (or analogous) motifs, and this may prove to be an important difference between the yeast and human systems.

Viral Late Domains and Vacuolar Protein Sorting

Our Vps4 dominant negative experiments indicate that different retroviruses (HIV-1 and MLV) that use different late domains (PTAP and PPPY, respectively) nevertheless both require a functional Vps pathway to bud. This suggests that the different primary cellular receptors for the different viral late domains may ultimately recruit

common factors downstream in the Vps pathway. Current evidence suggests that viruses that bud via PY late domains interact with members of the Nedd4 family of proteins (Garnier et al., 1996; Harty et al., 2000; Strack et al., 2000). A well-established cellular role for Nedd4 proteins is to ubiquitinate cell surface receptors and thereby target them for downregulation via endocytosis and lysosomal degradation (Hicke, 1999; Rotin et al., 2000). Nedd4 has multiple WW domains that recognize PY-like motifs on receptors (as well as on viral late domains), and a Hect E3 ubiquitin ligase domain that transfers ubiquitin onto the receptor (Harvey and Kumar, 1999). Importantly, Rsp5 (the yeast ortholog of Nedd4) has recently been shown to function both in the early stages of endocytosis *and* in downstream events involved in late endosomal trafficking (Dunn and Hicke, 2001; Wang et al., 2001). Thus, it appears that Nedd4 and Tsg101 may *both* help select cargo for incorporation into multivesicular bodies, perhaps performing complementary and/or synergistic roles in this process. One possibility is that Nedd4 conjugates ubiquitin onto target proteins and Tsg101 acts as a receptor for the ubiquitinated proteins.

In support of complementary roles for Tsg101 and Nedd4, many viral coat proteins contain consensus binding sites for *both* Tsg101 (P(T/S)AP) and Nedd4 (PPXY) (Strack et al., 2000). Similarly, there are also cellular proteins with consensus binding sites for both proteins. One class of proteins that carry both P(T/S)AP and PPXY motifs are surface receptors known to be degraded via the Vps pathway (e.g., connexins 43 and 45) (Berthoud et al., 2000; Strack et al., 2000). A second class are proteins that actually appear to *function* in the Vps pathway. One such example is *h*epatocyte growth factor-*r*egulated tyrosine kinase *s*ubstrate (Hrs, a homolog of yeast Vps27). Murine cells lacking Hrs exhibit abnormal endosomal morphologies, and the protein interacts with Nexin 1 (a homolog of yeast Vps5) and thereby helps regulate lysosomal targeting of the EGF receptor (Chin et al., 2001; Komada and Kitamura, 2001). However, not all PTAP and PPPY motifs appear to function in pairs, suggesting that other sequence motifs (in addition to PPXY) can mark proteins for ubiquitination and entry into the Vps pathway and that other recognition motifs (in addition to PTAP) can target proteins for MVB packaging.

A Potential Role for MVB Machinery in Virus Budding

Our studies suggest that HIV-1 Gag may bind Tsg101 and thereby usurp cellular machinery normally used for MVB formation to facilitate virus budding. The role of Tsg101 in virus budding could be analogous to the protein's normal cellular function of coordinating cargo selection and MVB formation, albeit at a different site—the plasma membrane. Several observations are consistent with this model. (1) As noted by Wills and colleagues (Patnaik et al., 2000), the topologies of viral budding and multivesicular body formation are similar: in both processes, the membrane invaginates *away from*, rather than into, the cytoplasm. Thus, all of the same cytoplasmic machinery used for MVB formation could, in principle, work in an analogous fashion to form viral particles at the plasma membrane. (2) As noted above, there is at least circumstantial evidence that two of the three proteins thought to function as late domain receptors (Tsg101 and Nedd4) may normally function in MVB cargo selection. Moreover, the cellular receptor for the YL late domain of EIAV appears to be the AP-50 subunit of AP-2 (Puffer et al., 1998), a complex that functions upstream in the pathway in selecting cargo for endocytosis (Owen and Luzio, 2000). (3) Finally, the observation that the ESCRT-1 complex requires substrate ubiquitination for MVB targeting (Katzmann et al., 2001), coupled with our observation that Tsg101 UEV domain has ubiquitin binding activity, suggests that during the budding process, Tsg101 may sense the ubiquitination state of a cellular protein or of Gag itself, thereby providing a rationale for the known requirement for ubiquitin in HIV-1 budding (Schubert et al., 2000b; Strack et al., 2000).

In summary, although the different late domain sequences of HIV-1 and MLV initially bind different cellular receptors, they may feed into common downstream steps of the Vps/MVB pathway. Indeed, given the unusual topology of MVB formation, it will not be surprising if many other enveloped viruses have evolved mechanisms for appropriating the MVB machinery to escape the cell.

Experimental Procedures

Yeast Two-Hybrid Experiments

Our yeast two-hybrid reagents and techniques have been reviewed (Bartel and Fields, 1997). Briefly, a bait construct expressing HIV-$1_{NYU/BR5}$ p6 (Gag residues 449–500) fused to the C terminus of Gal4 DNA binding domain (residues 1–147) was transformed into yeast strain PNY200 (*MATα trp1-901 leu2-3,112 ura3-52 his3-200 ade2 gal4Δ gal80*). Prey constructs expressing cDNA from human spleen poly(A)$^+$ RNA (Clontech) fused to the C terminus of the Gal4 activation domain (residues 768–881) were transformed into the yeast strain BK100 (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80 LYS2::GAL-HIS3 GAL2-ADE2 met2::GAL7-lacZ*), which incorporates the multiple reporter system (James et al., 1996). PNY200 cells (bait) were mated with BK100 cells (prey), and diploid yeast cells were selected in the presence of 3 mM 3-amino-1,2,4-triazole for the ability to synthesize tryptophan (bait), leucine (prey), histidine (bait/prey interaction), and adenine (bait/prey interaction). Approximately 4.2 million bait/prey pairs were tested, and prey encoding Tsg101 residues 7–390 were isolated twice (Tsg101 NCBI accession # NM_006292).

Interactions were confirmed by transforming bait and prey constructs into naïve yeast cells and performing liquid culture β-galactosidase assays. Cultures were grown overnight in synthetic media (−Leu, −Trp, + glucose) in 96-well plates, normalized for optical density, and lysed by addition of 6× lysis/substrate solution in 6× Z-buffer (60 mM KCl, 6 mM $MgSO_4$, 360 mM Na_2HPO_4, 240 mM NaH_2PO_4, 6 mg/ml CPRG, 0.12 U/ml lyticase, and 0.075% NP-40). Cultures were incubated for 2 hr at 37°C and clarified by centrifugation, and the optical absorbance of each supernatant was measured (575 nm). Significant responses were observed for p6 binding to full-length Tsg101 (>300-fold over background) and an N-terminal Tsg101 construct (residues 1–207; >30-fold over background), but not to a C-terminal Tsg101 construct (residues 207–390).

Recombinant Proteins

DNA encoding Tsg101 residues 1–145 (the UEV domain) was cloned into pET11d (Novagene), expressed in BL21(DE3) *E. coli* cells, and purified by conventional chromatography. Full details of the purification procedure will be published elsewhere (O.W.P. et al., in preparation). Expression of mutant GST-$p6_{NL4-3}$ and GST-Ub proteins followed the procedures described for wt GST-p6 (Jenkins et al., 2001).

Surface Plasmon Resonance

Surface plasmon resonance measurements were performed at 20°C using a BIACORE 3000 (Biacore AB, Uppsala, Sweden) equipped with a research-grade CM5 sensor chip. ~10 kRU anti-GST Ab was immobilized on all four flow cells using traditional amine-coupling chemistry (Johnsson et al., 1991). Soluble lysates from *E. coli* expressing GST-p6 proteins were diluted 10× in running buffer (20 mM Na_3PO_4, 150 mM NaCl, 0.005% P20, and 50 μg/mL BSA [pH 7.2]) and captured individually on three of the antibody surfaces at densities of 1–2.5 kRU. ~2 kRU recombinant GST protein was captured on the fourth (reference) antibody surface.

Tsg101 UEV in running buffer was injected in triplicate over the four flow cells at concentrations of 0, 0.09, 0.27, 0.82, 2.5, 7.4, 22, 67, and 200 μM (50 μL/min). Data were collected at a rate of 2 Hz during the 30 s association and dissociation phases. All Tsg 101/p6 interactions reached equilibrium rapidly and dissociated completely within seconds. To obtain equilibrium dissociation constants, the responses at t = 25 s were fit to simple 1:1 interaction binding isotherms (Myszka, 1999).

Plasmid Constructs

Vector R9 contains a full-length HIV-1_{NL4-3} expression clone (von Schwedler et al., 1998, and references therein). Kunkel mutagenesis was used to mutate the HIV-1 Gag p6 PTAP sequence to LIRL without disrupting the overlapping pol reading frame (Huang et al., 1995). R9ΔEnv and R9ΔPR were gifts from Chris Aiken, Vanderbilt University. pGag-GFP contains the rev-independent HIV-1_{HXB2} Gag sequence fused to EGFP, and was a gift from Marilyn Resh (Hermida-Matsumoto and Resh, 2000). The PTAP sequence of Gag p6 was also mutated to LIRL in this construct. The packaging vector pCLeco was used for MLV production (Naviaux et al., 1996). The MLV Gag p12 sequence PPPY was mutated to AAAA (Yuan et al., 1999) by PCR megaprimer mutagenesis of pCLeco. DNA encoding full-length Tsg101 was fused to the FLAG coding sequence using PCR and cloned into pIRES2-EGFP (Clontech). Seven silent mutations that render Tsg101-FLAG resistant to siRNA (denoted Tsg*-FLAG in Figure 2) were introduced into the wild-type Tsg101 coding region $_{413}$AACCTCCAGTCTTCTCTCGTC$_{433}$ using Kunkel mutagenesis (mutated nucleotides are underlined). The complete 437 aa reading frame of Vps4 was repaired and amplified from ATCC #81449, and cloned into pEGFP-C1 (Clontech) to create a GFP-Vps4 fusion protein. ATPase point mutations K173Q and E228Q were introduced by PCR megaprimer mutagenesis. Cloning details are available upon request.

Small Interfering RNA (siRNA)

21 nt RNA duplexes with symmetric 2 nt 3′ (2′-deoxy) thymidine overhangs corresponding to *Tsg101* coding nt 413–433 were synthesized and HPLC purified. RNA sequences: Sense, 5′ CCU CCA GUC UUC UCU CGU CTT; Antisense, 5′ GAC GAG AGA AGA CUG GAG GTT; Inverted sense, 5′ CUG CUC UCU UCU GAC CUC CTT; Inverted antisense, GGA GGU CAG AAG AGA GCA GTT. Oligonucleotides were annealed as described (Elbashir et al., 2001).

Protein Expression in 293T and T Cells

293T cells were cotransfected with the indicated amounts of R9 and Vps4 expression vectors in 6 well plates using the calcium phosphate method as described (von Schwedler et al., 1998). All other transfections were performed as described in the text using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells and supernatants were harvested 24–72 hr post-transfection as described in the text.

Western Blots

Cytoplasmic and sucrose-pelleted viral lysates were resolved by SDS-PAGE and blotted for ECL as described (von Schwedler et al., 1998). The following primary antibodies were used: rabbit anti-HIV CA antibody from Hans-Georg Krausslich, Heidelberg, Germany (at 1:2000); rabbit anti-HIV MA from Didier Trono, Geneva, Switzerland (at 1:50,000); murine monoclonal anti-Tsg101-4A10 from GeneTex, Inc. (at 1:1000), murine monoclonal anti-FLAG M2 from Sigma (at 1:3000), rabbit anti-14-3-3β K19 from Santa Cruz Biotechnology (at 1:3000), anti-GFP-HRP from Clontech (at 1:500), and goat anti-MLV p30 from John Elder, Scripps Institute, La Jolla (at 1:1000).

Viral Replication Assays

Infectivity of HIV-1 released into the supernatants from cells transfected with R9 constructs was assayed by MAGIC assay in P4 cells as described (von Schwedler et al., 1998, and references therein), except that infections were performed at 3 different dilutions in triplicate in 48-well plates. Blue cells and syncytia were counted 2 days after infection. MLV infectivity was assayed by packaging pCL-lacZ in the M-MLV retroviral vector pCLeco (Naviaux et al., 1996), transducing NIH3T3 cells, and staining cells for β-galactosidase activity 2 days later, as described (von Schwedler et al., 1998).

Electron Microscopy

Transfected 293T cells were fixed with 2.5% glutaraldehyde/1% paraformaldehyde for 30 min and stained as described (von Schwedler et al., 1998), except that samples were dehydrated in ethanol and embedded in Spurr's plastic.

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References

Amerik, A.Y., Nowak, J., Swaminathan, S., and Hochstrasser, M. (2000). The doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways. Mol. Biol. Cell *11*, 3365–3380.

Babst, M., Wendland, B., Estepa, E.J., and Emr, S.D. (1998). The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. EMBO J. *17*, 2982–2993.

Babst, M., Odorizzi, G., Estepa, E.J., and Emr, S.D. (2000). Mammalian tumor susceptibility gene 101 (TSG101) and the yeast homologue, Vps23p, both function in late endosomal trafficking. Traffic *1*, 248–258.

Bartel, P.L., and Fields, S. (1997). The Yeast Two Hybrid Screen (New York: Oxford University Press, Inc).

Berthoud, V.M., Tadros, P.N., and Beyer, E.C. (2000). Connexin and gap junction degradation. Methods *20*, 180–187.

Bishop, N., and Woodman, P. (2000). ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking. Mol. Biol. Cell *11*, 227–239.

Bishop, N., and Woodman, P. (2001). TSG101/mammalian VPS23 and mammalian VPS28 interact directly and are recruited to VPS4-induced endosomes. J. Biol. Chem. *276*, 11735.

Chin, L.S., Raynor, M.C., Wei, X., Chen, H.Q., and Li, L. (2001). Hrs interacts with sorting nexin 1 and regulates degradation of epidermal growth factor receptor. J. Biol. Chem. *276*, 7069–7078.

Craven, R.C., Harty, R.N., Paragas, J., Palese, P., and Wills, J.W. (1999). Late domain function identified in the vesicular stomatitis virus M protein by use of rhabdovirus-retrovirus chimeras. J. Virol. *73*, 3359–3365.

Dunn, R., and Hicke, L. (2001). Domains of the rsp5 ubiquitin-protein ligase required for receptor-mediated and fluid-phase endocytosis. Mol. Biol. Cell *12*, 421–435.

Dupre, S., and Haguenauer-Tsapis, R. (2001). Deubiquitination step in the endocytic pathway of yeast plasma membrane proteins: crucial role of doa4p ubiquitin isopeptidase. Mol. Cell. Biol. *21*, 4482–4494.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K.,

and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494–498.

Freed, E.O. (1998). HIV-1 gag proteins: diverse functions in the virus life cycle. Virology *251*, 1–15.

Galan, J.M., Wiederkehr, A., Seol, J.H., Haguenauer-Tsapis, R., Deshaies, R.J., Riezman, H., and Peter, M. (2001). Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. Mol. Cell. Biol. *21*, 3105–3117.

Garnier, L., Wills, J.W., Verderame, M.F., and Sudol, M. (1996). WW domains and retrovirus budding. Nature *381*, 744–745.

Göttlinger, H.G., Dorfman, T., Sodroski, J.G., and Haseltine, W.A. (1991). Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc. Natl. Acad. Sci. USA *88*, 3195–3199.

Harty, R.N., Paragas, J., Sudol, M., and Palese, P. (1999). A proline-rich motif within the matrix protein of vesicular stomatitis virus and rabies virus interacts with WW domains of cellular proteins: implications for viral budding. J. Virol. *73*, 2921–2929.

Harty, R.N., Brown, M.E., Wang, G., Huibregtse, J., and Hayes, F.P. (2000). A PPxY motif within the VP40 protein of ebola virus interacts physically and functionally with a ubiquitin ligase: implications for filovirus budding. Proc. Natl. Acad. Sci. USA *97*, 13871–13876.

Harvey, K.F., and Kumar, S. (1999). Nedd4-like proteins: an emerging family of ubiquitin-protein ligases implicated in diverse cellular functions. Trends Cell Biol. *9*, 166–169.

Hermida-Matsumoto, L., and Resh, M.D. (2000). Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. J. Virol. *74*, 8670–8679.

Hicke, L. (1999). Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. Trends Cell Biol. *9*, 107–112.

Huang, M., Orenstein, J.M., Martin, M.A., and Freed, E.O. (1995). p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. J. Virol. *69*, 6810–6818.

James, P., Halladay, J., and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two- hybrid selection in yeast. Genetics *144*, 1425–1436.

Jayakar, H.R., Murti, K.G., and Whitt, M.A. (2000). Mutations in the PPPY motif of vesicular stomatitis virus matrix protein reduce virus budding by inhibiting a late step in virion release. J. Virol. *74*, 9818–9827.

Jenkins, Y., Pornillos, O., Rich, R., Myszka, D., Sundquist, W., and Malim, M. (2001). Biochemical analyses of the interactions between HIV-1 Vpr and p6Gag. J. Virol., in press.

Johnsson, B., Lofas, S., and Lindquist, G. (1991). Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. Anal. Biochem. *198*, 268–277.

Katzmann, D., Babst, M., and Emr, S.D. (2001). Uniquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein complex, ESCRT-1. Cell *106*, 145–155.

Komada, M., and Kitamura, N. (2001). Hrs and hbp: possible regulators of endocytosis and exocytosis. Biochem. Biophys. Res. Commun. *281*, 1065–1069.

Koonin, E.V., and Abagyan, R.A. (1997). TSG101 may be the prototype of a class of dominant negative ubiquitin regulators. Nat. Genet. *16*, 330–331.

Lemmon, S.K., and Traub, L.M. (2000). Sorting in the endosomal system in yeast and animal cells. Curr. Opin. Cell Biol. *12*, 457–466.

Li, L., and Cohen, S.N. (1996). Tsg101: a novel tumor susceptibility gene isolated by controlled homozygous functional knockout of allelic loci in mammalian cells. Cell *85*, 319–329.

Li, Y., Kane, T., Tipper, C., Spatrick, P., and Jenness, D.D. (1999). Yeast mutants affecting possible quality control of plasma membrane proteins. Mol. Cell. Biol. *19*, 3588–3599.

Li, L., Liao, J., Ruland, J., Mak, T.W., and Cohen, S.N. (2001). A TSG101/MDM2 regulatory loop modulates MDM2 degradation and MDM2/p53 feedback control. Proc. Natl. Acad. Sci. USA *98*, 1619–1624.

Losko, S., Kopp, F., Kranz, A., and Kolling, R. (2001). Uptake of the ATP-binding cassette (ABC) transporter Ste6 into the yeast vacuole is blocked in the doa4 Mutant. Mol. Biol. Cell *12*, 1047–1059.

Myszka, D.G. (1999). Improving biosensor analysis. J. Mol. Recognit. *12*, 279–284.

Naviaux, R.K., Costanzi, E., Haas, M., and Verma, I.M. (1996). The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. J. Virol. *70*, 5701–5705.

Ott, D.E., Coren, L.V., Chertova, E.N., Gagliardi, T.D., and Schubert, U. (2000). Ubiquitination of HIV-1 and MuLV Gag. Virology *278*, 111–121.

Owen, D.J., and Luzio, J.P. (2000). Structural insights into clathrin-mediated endocytosis. Curr. Opin. Cell Biol. *12*, 467–474.

Parent, L.J., Bennett, R.P., Craven, R.C., Nelle, T.D., Krishna, N.K., Bowzard, J.B., Wilson, C.B., Puffer, B.A., Montelaro, R.C., and Wills, J.W. (1995). Positionally independent and exchangeable late budding functions of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. J. Virol. *69*, 5455–5460.

Patnaik, A., Chau, V., and Wills, J.W. (2000). Ubiquitin is part of the retrovirus budding machinery. Proc. Natl. Acad. Sci. USA *97*, 13069–13074.

Ponting, C.P., Cai, Y.D., and Bork, P. (1997). The breast cancer gene product TSG101: a regulator of ubiquitination? J. Mol. Med. *75*, 467–469.

Puffer, B.A., Parent, L.J., Wills, J.W., and Montelaro, R.C. (1997). Equine infectious anemia virus utilizes a YXXL motif within the late assembly domain of the Gag p9 protein. J. Virol. *71*, 6541–6546.

Puffer, B.A., Watkins, S.C., and Montelaro, R.C. (1998). Equine infectious anemia virus Gag polyprotein late domain specifically recruits cellular AP-2 adapter protein complexes during virion assembly. J. Virol. *72*, 10218–10221.

Rotin, D., Staub, O., and Haguenauer-Tsapis, R. (2000). Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. J. Membr. Biol. *176*, 1–17.

Ruland, J., Sirard, C., Elia, A., MacPherson, D., Wakeham, A., Li, L., Luis De La Pompa, J., Cohen, S.N., and Mak, T.W. (2001). p53 Accumulation, defective cell proliferation, and early embryonic lethality in mice lacking tsg101. Proc. Natl. Acad. Sci. USA *98*, 1859–1864.

Schubert, U., Anton, L.C., Gibbs, J., Norbury, C.C., Yewdell, J.W., and Bennink, J.R. (2000a). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature *404*, 770–774.

Schubert, U., Ott, D.E., Chertova, E.N., Welker, R., Tessmer, U., Princiotta, M.F., Bennink, J.R., Krausslich, H.G., and Yewdell, J.W. (2000b). Proteasome inhibition interferes with gag polyprotein processing, release, and maturation of HIV-1 and HIV-2. Proc. Natl. Acad. Sci. USA *97*, 13057–13062.

Shih, S.C., Sloper-Mould, K.E., and Hicke, L. (2000). Monoubiquitin carries a novel internalization signal that is appended to activated receptors. EMBO J. *19*, 187–198.

Strack, B., Calistri, A., Accola, M.A., Palu, G., and Göttlinger, H.G. (2000). A role for ubiquitin ligase recruitment in retrovirus release. Proc. Natl. Acad. Sci. USA *97*, 13063–13068.

VanDemark, A.P., Hofmann, R.M., Tsui, C., Pickart, C.M., and Wolberger, C. (2001). Molecular insights into polyubiquitin chain assembly: Crystal structure of the Mms2/Ubc13 heterodimer. Cell *105*, 711–720.

VerPlank, L., Bouamr, F., LaGrassa, T.J., Agresta, B., Kikonyogo, A., Leis, J., and Carter, C.A. (2001). Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55Gag. Proc. Natl. Acad. Sci. USA *98*, 7724–7729.

Vogt, V.M. (2000). Ubiquitin in retrovirus assembly: actor or bystander? Proc. Natl. Acad. Sci. USA *97*, 12945–12947.

von Schwedler, U.K., Stemmler, T.L., Klishko, V.Y., Li, S., Albertine, K.H., Davis, D.R., and Sundquist, W.I. (1998). Proteolytic refolding of the HIV-1 capsid protein amino-terminus facilitates viral core assembly. EMBO J. *17*, 1555–1568.

Wang, G., McCaffery, J.M., Wendland, B., Dupre, S., Haguenauer-Tsapis, R., and Huibregtse, J.M. (2001). Localization of the Rsp5p ubiquitin-protein ligase at multiple sites within the endocytic pathway. Mol. Cell. Biol. *21*, 3564–3575.

Wills, J.W., and Craven, R.C. (1991). Form, function, and use of retroviral gag proteins. AIDS *5*, 639–654.

Xie, W., Li, L., and Cohen, S.N. (1998). Cell cycle-dependent subcellular localization of the TSG101 protein and mitotic and nuclear abnormalities associated with TSG101 deficiency. Proc. Natl. Acad. Sci. USA *95*, 1595–1600.

Yuan, B., Li, X., and Goff, S.P. (1999). Mutations altering the moloney murine leukemia virus p12 Gag protein affect virion production and early events of the virus life cycle. EMBO J. *18*, 4700–4710.

Yuan, B., Campbell, S., Bacharach, E., Rein, A., and Goff, S.P. (2000). Infectivity of moloney murine leukemia virus defective in late assembly events is restored by late assembly domains of other retroviruses. J. Virol. *74*, 7250–7260.

Zhong, Q., Chen, Y., Jones, D., and Lee, W.H. (1998). Perturbation of TSG101 protein affects cell cycle progression. Cancer Res. *58*, 2699–2702.