HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress

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Retroviral Gag proteins encode sequences, termed late domains, which facilitate the final stages of particle budding from the plasma membrane. We report here that interactions between Tsg101, a factor involved in endosomal protein sorting, and short peptide motifs in the HIV-1 Gag late domain and Ebola virus matrix (EbVp40) proteins are essential for efficient egress of HIV-1 virions and Ebola virus-like particles. EbVp40 recruits Tsg101 to sites of particle assembly and a short, EbVp40-derived Tsg101-binding peptide sequence can functionally substitute for the HIV-1 Gag late domain. Notably, recruitment of Tsg101 to assembling virions restores budding competence to a late-domain–defective HIV-1 in the complete absence of viral late domain. These studies define an essential virus–host interaction that is conserved in two unrelated viruses. Because the Tsg101 is recruited by small, conserved viral sequence motifs, agents that mimic these structures are potential inhibitors of the replication of these lethal human pathogens.

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An essential step in the life cycle of enveloped viruses is a budding process that occurs at either intracellular or plasma membranes. Thus, the separation of the nascent virion from the host cell requires a membrane fusion event that could, in principle, be either spontaneous or mediated by viral and/or cellular factors. Studies on the morphogenesis of retroviral particles indicate that many encode a so-called late or 'L'-domain within the Gag protein whose disruption results in a phenotype characterized by virion assembly that is normal except for this late budding event[1–6]. Specifically, L-domain mutant virions accumulate at the plasma membrane and remain tethered to the infected cell by a membranous 'stalk'. L-domains are largely transferable between different retroviruses and can exert their activity when positioned at a variety of locations within the Gag protein[4,7]. This, in itself, suggests that L-domains act by recruiting additional factors rather than by altering the conformation of the retroviral Gag protein or otherwise directly affecting virion morphogenesis.

Each of the retroviral L-domains characterized contains one of three essential sequence motifs, PT/SAP, PPXY or YXXL (refs. 2–6,8). These sequence motifs have been reported to constitute at least part of a binding site for cellular proteins that may be involved in particle budding, including Tsg101, Nedd4-like ubiquitin ligases and AP2 (Refs. 9–12). HIV-1 particle budding is dependent on a PTAP motif, present within the p6[Gag] protein and conserved in all HIV and SIV strains[6,8]. Recently, HIV-1 p6 was reported to bind Tsg101, a component of the vesicular protein-sorting machinery[13–15], although the functional significance of this interaction is not established[11]. In this report, we present compelling evidence that short PTAP-containing peptide sequences both in HIV-1 Gag and in the matrix protein (Vp40) of the otherwise unrelated Ebola virus are necessary and sufficient to recruit Tsg101 to sites of particle assembly. In addition, recruitment of Tsg101 is necessary for efficient formation of HIV-1 virions or Ebola Vp40 virus–like particles. Notably, Tsg101 recruitment to the assembling virion in *trans*, mediated either by short viral peptides or in the complete absence of a viral L-domain, restores budding competence to an L-domain–defective HIV-1.

Functional significance of the HIV-1 Gag–Tsg101 interaction

To determine whether the Gag–Tsg101 interaction has an important role in HIV-1 virion egress, we first carried out a correlative mutagenesis experiment by introducing a series of missense mutations (M1–M7, Fig. 1*a*) into the p6 protein. These mutations span the PTAP motif, which is known to be essential for L-domain function, and were selected so as not to alter the coding potential of the underlying *pol* gene. We examined each mutant Gag for its ability to interact with Tsg101 in a yeast two-hybrid assay and to generate extracellular virion when expressed in a proviral context. These experiments revealed an essentially perfect correlation between the ability of HIV-1 Gag to bind Tsg101 and to participate in the formation of infectious extracellular virions (Fig. 1). Specifically, although each mutant was expressed approximately equivalently in yeast, alteration of any single residue in a contiguous four-residue sequence of amino acids (P_7-T_8-A_9-P_{10}) completely ablated the Gag–Tsg101 interaction (Fig. 1*b*). In each case, this phenotype was accompanied by a viral processing defect in mammalian cells that included the accumulation of late Gag processing intermediates, a characteristic of L-domain–defective HIV-1 mutants[8] (Fig. 1*c*). In addition, proviruses encoding each of the mutant Gag proteins (M2–M5) that did not bind Tsg101 generated very small amounts of virions, as measured by ELISA or infectivity assays (Fig. 1*c* and *d*). In contrast, alteration of residues immediately flanking the P_7-T_8-A_9-P_{10} sequence (in M1, M6 and M7) resulted in only minor defects in both Tsg101 binding and virion production (Fig. 1*a–c*). Thus, these experiments establish the functional significance of the Tsg101–HIV-1 Gag interaction and define a four-residue sequence within p6 that is required for both Tsg101 binding and virion egress.

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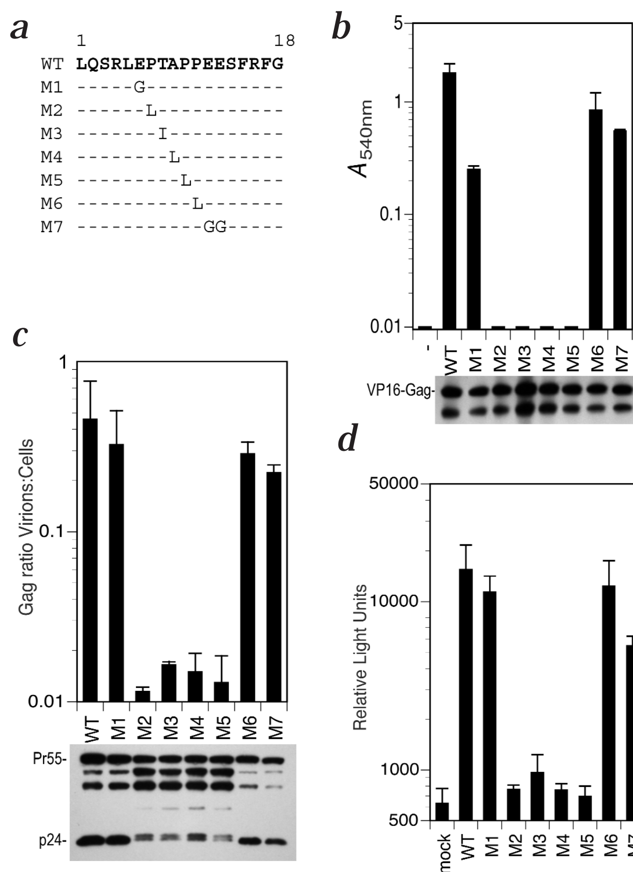

Fig. 1 Correlative mutagenesis establishes the functional importance of the HIV-1 Gag–Tsg101 interaction. ***a***, Mutations introduced into HIV-1 p6 spanning the PTAP motif. ***b***, Interaction between GAL4-Tsg101 and wild-type (WT) or mutant VP16-Gag fusion proteins using the yeast two-hybrid assay. '–', yeast transformed with GAL4-Tsg101 and VP16 (without a fused protein) expression plasmids. Expression of the wild-type and mutant VP16-Gag proteins verified by western-blot analysis with a p24-specific monoclonal antibody (lower panel). ***c***, Virion production by HeLa cells transfected with HIV-1 proviral plasmids bearing the p6 mutations shown in (*a*). Results are expressed as the ratio of Gag protein present in virion and cell lysates. Western-blot analysis of Gag in transfected cells is shown in the lower panel. ***d***, Infectious virus production by cells transfected with wild-type and mutant proviruses, determined using P4/R5 cells. 'Mock', β-gal activity in uninfected cells.

A Tsg101 binding motif mediates Ebola virus-like particle egress

Several enveloped viruses contain PTAP or PPXY sequences that conform to the essential L-domain sequence motifs found in retroviruses[9,16–18]. In some instances, both motifs are present within a relatively short linear sequence. The Ebola virus Vp40 matrix protein (EbVp40) is unusual in that it contains overlapping PTAP and PPXY motifs near its amino (N) terminus, within the sequence $P_7TAPPEY_{13}$ (Fig. 2*a*). Expression of EbVp40 in mammalian cells is sufficient to generate extracellular particles that lack other Ebola virus proteins but whose morphology otherwise resembles that of authentic filovirus virions[19]. There is evidence that the PPXY motif–dependent interaction with Nedd4 is essential to this property[16,19,20], but a possible role for the PTAP motif in particle egress has not been determined.

We first examined the ability of an EbVp40 protein bearing a single amino-acid substitution within the PTAP motif (P7L), but containing an intact PPXY motif (Fig. 2*a*), to generate extracellular particles. In fact, the mutant EbVp40 (P7L) protein was expressed at an amount equivalent to that of the wild-type protein but exhibited a substantial defect in extracellular particle formation (Fig. 2*b*). In addition, the magnitude of the defect was at least as great as that described for a EbVp40 deletion mutant lacking the entire N-terminal 31-amino-acid stretch (that is, both the PTAP and the PPXY motifs)[19].

This experiment showed that an intact PTAP sequence motif is required for EbVp40 particle production. However, if the PTAP motif within EbVp40 exerts an enhancing effect on particle formation by the same mechanism as that for HIV-1 p6, then EbVp40 should also bind Tsg101. In fact, in a yeast two-hybrid assay, EbVp40 bound Tsg101 at least as efficiently as HIV-1 Gag (Fig. 2*c*). In addition, the single amino-acid substitution within PTAP (P7L) that attenuated extracellular particle formation also ablated the Tsg101–EbVp40 interaction, without affecting the level of EbVp40 expression in yeast (Fig. 2*c* and *d*). Thus, in both HIV-1 Gag and EbVp40, a PTAP sequence motif is required for interaction with Tsg101 and efficient particle egress.

EbVp40 recruits Tsg101 to the plasma-membrane

If recruitment of Tsg101 is the mechanism by which the EbVp40 PTAP motif enhances particle egress, we reasoned that it might be possible to observe relocalization of Tsg101 to sites of particle assembly in EbVp40 expressing cells. EbVp40 localizes to the plasma membrane[19] before budding into the extracellular medium. Therefore, we examined the localization of a myc epitope–tagged Tsg101 protein, either expressed alone or in the presence of wild-type or P7L mutant forms of HA-tagged EbVp40 (Fig. 3). In the absence of EbVp40, myc-Tsg101 exhibited a punctate distribution, predominantly within the cell cytoplasm.

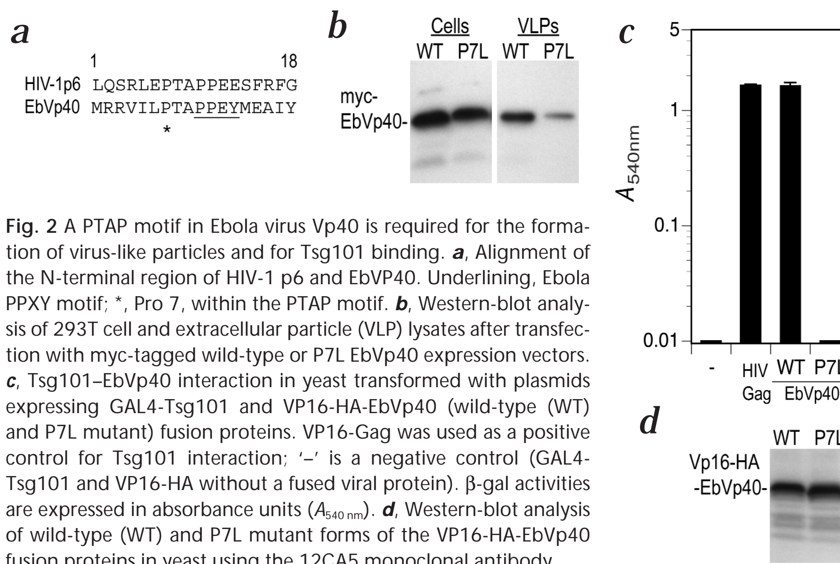

Fig. 2 A PTAP motif in Ebola virus Vp40 is required for the formation of virus-like particles and for Tsg101 binding. ***a***, Alignment of the N-terminal region of HIV-1 p6 and EbVP40. Underlining, Ebola PPXY motif; *, Pro 7, within the PTAP motif. ***b***, Western-blot analysis of 293T cell and extracellular particle (VLP) lysates after transfection with myc-tagged wild-type or P7L EbVp40 expression vectors. ***c***, Tsg101–EbVp40 interaction in yeast transformed with plasmids expressing GAL4-Tsg101 and VP16-HA-EbVp40 (wild-type (WT) and P7L mutant) fusion proteins. VP16-Gag was used as a positive control for Tsg101 interaction; '–' is a negative control (GAL4-Tsg101 and VP16-HA without a fused viral protein). β-gal activities are expressed in absorbance units ($A_{540\,nm}$). ***d***, Western-blot analysis of wild-type (WT) and P7L mutant forms of the VP16-HA-EbVp40 fusion proteins in yeast using the 12CA5 monoclonal antibody.

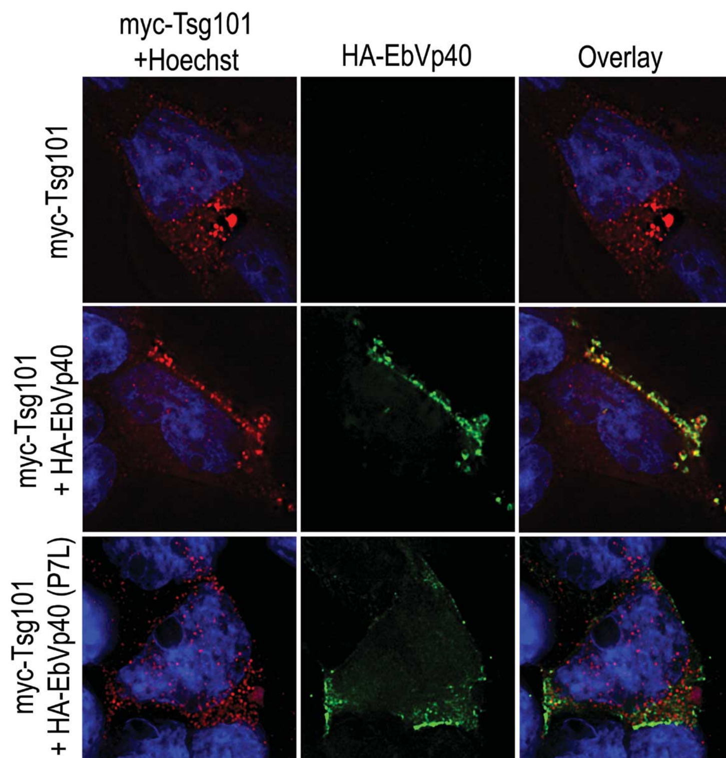

Fig. 3 EbVp40 induces the relocalization of Tsg101 to sites of particle assembly. 293T cells transfected with a myc-tagged Tsg101 (red) expression plasmid in the absence or the presence of wild-type and P7L mutant forms of HA-tagged EbVp40 (green), as indicated on the left of the figure. Nuclei are stained blue. Hoechst, Hoechst 33258. Images represent single 200-nm-thick optical sections. Magnification, x100.

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Upon co-expression with HA-EbVp40, however, we observed recruitment of myc-Tsg101 to the plasma membrane, where it substantially colocalized with EbVp40 (Fig. 3). Notably, co-expression with the P7L mutant form of EbVp40 did not induce redistribution of Tsg101. This was not due to aberrant viral protein localization, because EbVp40(P7L), like the wild-type protein, also accumulated at the plasma membrane (Fig. 3). Thus, EbVp40 recruits Tsg101 to sites of particle budding in a PTAP-dependent manner.

A Tsg101 binding peptide constitutes a functional L-domain

Because there is virtually no sequence homology between HIV-1 Gag and EbVp40 outside the PTAP motif, we next asked whether short viral amino-acid sequences containing this motif are sufficient to recruit Tsg101. In fact, 10 residues derived from HIV-1 p6 or 12 residues derived from EbVp40 bound Tsg101 in the yeast two-hybrid assay, albeit slightly less efficiently than did the full-length HIV-1 Gag or EbVp40 proteins (Fig. 4*a*). Given that both EbVp40- and HIV-1–derived sequences bound Tsg101, it follows that the EbVp40 sequence should be able to substitute functionally for the HIV-1 sequence if this interaction is the mechanism by which they exert their effects. We therefore substituted 10 residues within HIV-1 p6 Gag with the equivalent residues of EbVp40 to generate pHIV/p6/ePTAP (Fig. 4*b*). This construct generated infectious HIV-1 particles as efficiently as the parental clone (Fig. 4*c*). In the HIV/p6/ePTAP provirus, however, both PTAP and PPXY motifs are present in the EbVp40-derived sequence. Therefore, to distinguish whether PTAP or PPXY motifs were important for L-domain function in this context, we introduced a single amino-acid substitution (P7L) that disrupts the PTAP but not the PPXY motif and is equivalent to that in the HIV-1 Gag M2 (Fig. 1) and the EbVp40(P7L) (Fig. 2) mutants. As was the case with HIV-1 Gag and EbVp40, this substitution entirely ablated the ability of HIV/p6/ePTAP to bind to Tsg101 (data not shown) and greatly reduced the formation of infectious virions (Fig. 4*c*). When compared with the same substitution in HIV-1 (M2), this effect was the same in magnitude and was accompanied by essentially identical effects on intracellular Gag processing and overall particle release (Fig. 4*d* versus Fig. 1). These findings show that the L-domain function provided by the short EbVp40-derived sequence is, at least in this context, entirely due to the PTAP motif and that the PPXY containing sequence is inactive in the absence of the overlapping PTAP motif.

A characteristic of retroviral L-domains is that they can act in a position-independent manner[4,7]. If the 12-residue, Tsg101-binding EbVp40-derived sequence is a true L-domain, it should be active when inserted into an alternative position within the HIV-1 Gag precursor. Therefore, we used a proviral construct, termed dGH, from which sequences encoding the globular head of the HIV-1 matrix protein had been deleted. This Gag domain is not required for efficient HIV-1 particle formation[21,22]. We replaced the deleted sequence with two copies of a sequence encoding either the 12-residue ePTAP sequence or, as a control, the influenza HA-epitope tag (Fig. 4*b*). As expected, the dGH(2xHA) provirus efficiently generated extracellular particles, but only when the p6 L-domain was intact (Fig. 4*e*). In contrast, the dGH/L$^-$(2xePTAP) provirus generated extracellular particles, in the absence of a functional p6 L-domain, at least as efficiently as dGH(2xHA). Thus, a 12-residue, filovirus-derived Tsg101-binding peptide sequence mediates particle formation when present near the HIV-1 Gag N or COOH (C) terminus and, therefore, has the position-independent activity characteristic of a retroviral L-domain.

Tsg101 recruitment is sufficient for L-domain function

These experiments indicated that both HIV-1 Gag and EbVp40 encode Tsg101 binding sequences that are required for the efficient egress of particles. We next investigated whether viral L-domains would function in *trans,* and, subsequently, whether recruitment of Tsg101 was sufficient to explain their activity (Fig. 5*a* and *b*). Initially, we determined whether an L-domain–defective provirus could be functionally *trans*-complemented by a provirus that was defective in another way. We cotransfected a L-domain–defective proviral plasmid, termed pL$^-$, in which the core PTAP motif is changed to LTAL with a second proviral plasmid, termed pG2A, that contains a functional L-domain but lacks a functional membrane binding domain; therefore this second provirus is also defective for virion production. Because the HIV-1 Gag protein efficiently multimerizes within cells[23,24], we reasoned that Gag complexes might form that contained both membrane-binding and L-domain functions (Fig. 5*a*). In fact, the amounts of viral particles harvested from cotransfected cells were significantly higher than when ei-

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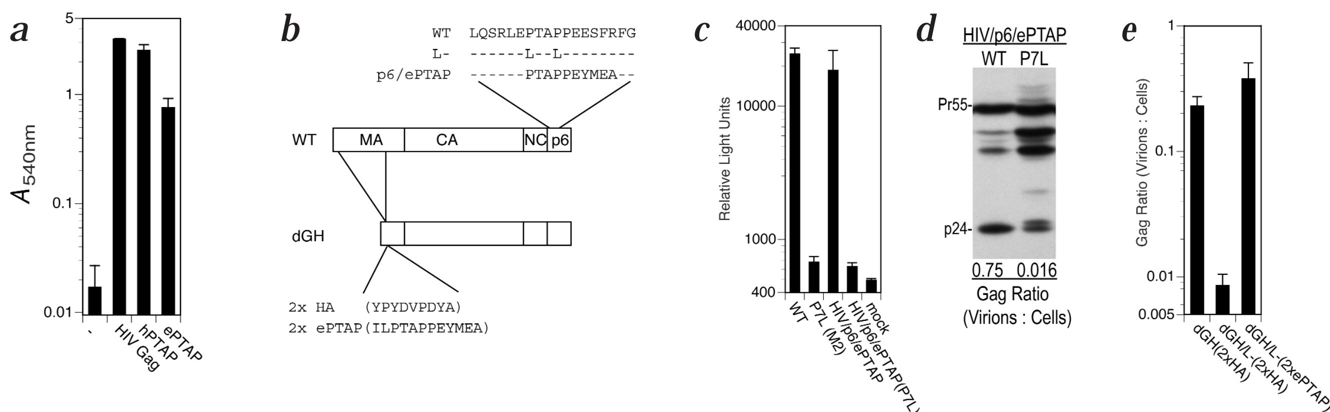

Fig. 4 Short sequences derived from HIV-1 p6 and EbVp40 and containing the PTAP motif bind Tsg101, and the EbVp40 sequence functionally substitutes for the HIV-1 L-domain. ***a***, GAL4-Tsg101 binding to VP16-hPTAP and VP16-ePTAP in yeast. VP16-Gag was used as a positive control for Tsg101 interaction. '–', unfused VP16 negative control. ***b***, Derivation of the proviral constructs pL⁻, pHIV/p6/ePTAP, dGH(2xHA) and dGH/L⁻(2xePTAP). ***c***, Infectious virus production by HeLa cells transfected with the HIV/p6/ePTAP and HIV/p6/ePTAP(P7L), the wild-type (WT) and P7L (M2) proviruses serve as controls. ***d***, Expression and processing of Gag by HIV/p6/ePTAP constructs as determined by western blot. Numbers below each lane indicate the ratio of the Gag concentrations in virion and cell lysates, determined by p24 ELISA. ***e***, Virion production by HeLa cells transfected with dGH-derived proviruses dGH(2xHA), dGH/L⁻(2xHA) and dGH/L⁻(2xePTAP).

ther provirus was transfected alone, as measured by ELISA (data not shown) or infectivity assays (Fig. 5*c*). Although this complementation was somewhat inefficient, in that it was difficult to detect virions by western blotting (Fig. 5*c*), this experiment did show that the HIV-1 L-domain could function in *trans*.

We next constructed an HIV-1 proviral plasmid, termed pENX, in which Gag is truncated at residue 6 of the p6 protein and the *pol*, *vif* and *vpr* sequences are also deleted and replaced by a short synthetic sequence for convenient insertion of candidate L-domains (Fig. 5*b*). Cells transfected with pENX express a truncated Gag precursor, which remains unprocessed and predominantly cell-associated because of the absence of an L-domain (data not shown).

We inserted a variety of sequences into pENX so that they would be expressed as proteins fused to the C terminus of the truncated Gag precursor (Fig. 5*c*). We examined whether these Gag fusion proteins could restore infectious virus production when co-expressed with pL⁻. In fact, pENX/p6 restored virion production very efficiently (Fig. 5*c*). We believe that the presence of a functional membrane-binding sequence in the pENX-derived Gag protein resulted in more efficient Gag multimerization at the plasma membrane[25] than occurred with pG2A. No complementation resulted when pENX was used in place of pENX/p6 or when the LTAL mutant form of p6 was substituted (in pENX/p6L⁻). The L-domain of a distantly related lentivirus, equine infectious anemia virus (EIAV), which is encoded within the p9 protein of EIAV Gag, also *trans*-complemented pL⁻ when expressed in the pENX context, although somewhat less efficiently than p6 (Fig. 5*c*).

The presence of a viral L-domain, expressed in *trans*, at the site of HIV-1 budding almost completely reversed the Gag processing defect that occurs within cells upon expression of the L-domain–defective provirus (Fig. 5*c*). In addition, the virion

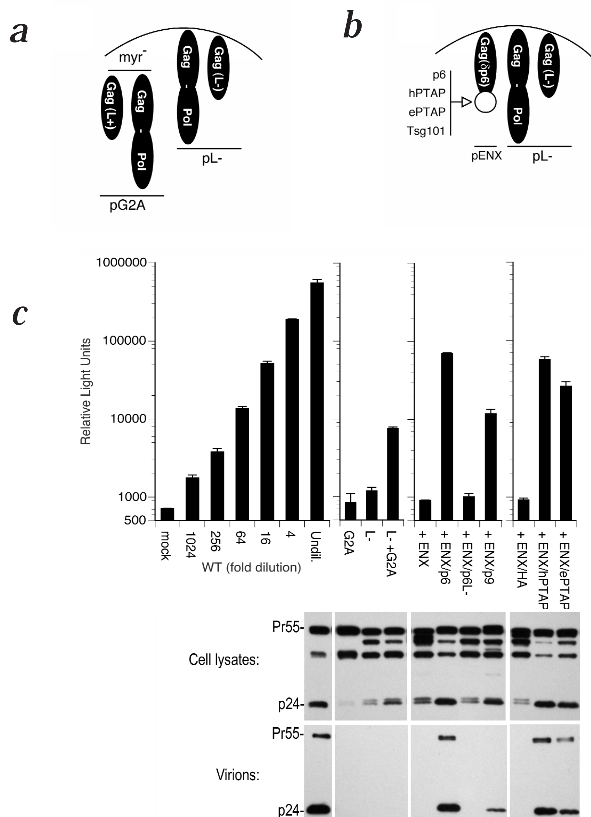

Fig. 5 An L-domain–defective HIV-1 can be complemented in *trans* by recruitment of homologous or heterologous late domains or Tsg101-binding peptides to the site of particle assembly. ***a***, Schema of the initial *trans*-complementation strategy. An L-domain–defective proviral plasmid (pL⁻) is co-expressed with a membrane binding–defective pG2A counterpart; Gag multimerization results in complementation of the defects. ***b***, Alternatively, a truncated Gag protein that lacks p6 is used to assess the ability of various sequences to restore egress of L-domain–defective virus. ***c***, *Trans*-complementation experiments in HeLa cells transfected either with a wild-type proviral plasmid (WT) or with pL⁻ and a pG2A or pENX construct. Upper panel, infectious virion production falls within the linear range of the assay shown in the leftmost chart. Lower panel, western-blot analysis of HIV-1 Gag in cell and virion lysates.

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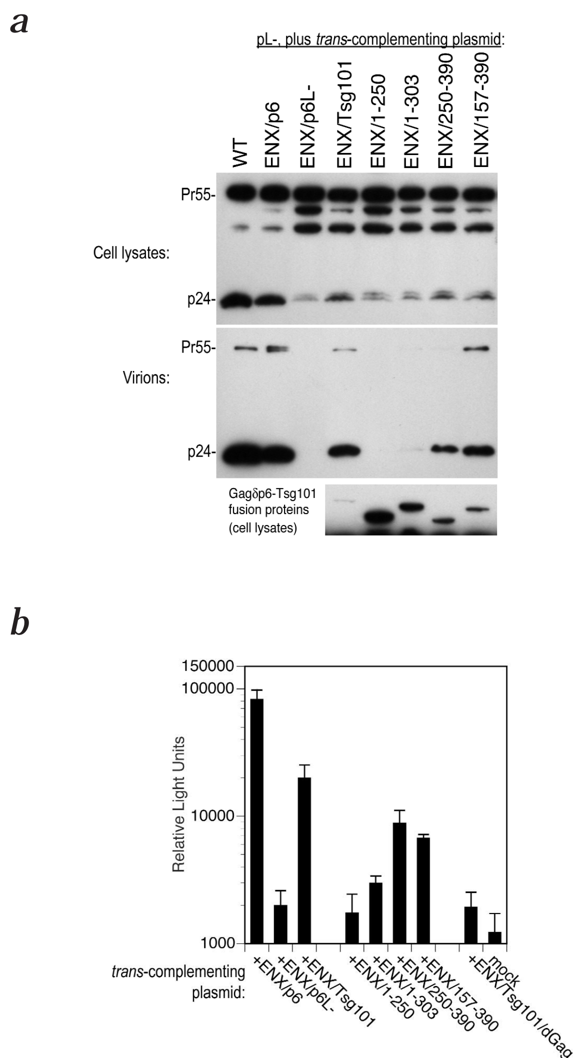

Fig. 6 Recruitment of Tsg101 to sites of HIV-1 particle assembly rescues virion budding in the absence of a viral L-domain. ***a***, Western-blot analysis of HIV-1 Gag in HeLa cell lysates and virions after transfection with either pWT or a combination of pL⁻ and a *trans*-complementing pENX-derived plasmid. The small lower panel is a longer exposure of the upper part of the gel to reveal expression of the pENX-derived Gagδp6-Tsg101 (and deletion mutant) in cell lysates. ***b***, Infectious virus production by HeLa cells transfected with pL⁻ and the pENX–derived plasmids.

particles harvested from these cotransfection/*trans*-complementation experiments contained Gag proteins that were efficiently processed and were also infectious (Fig. 5*c*). Thus, the viral L-domains, expressed in a context in which *pol* is not present in *cis*, are able to complement a viral construct in *trans* that contains *pol* but lacks a functional L-domain.

Because the 10- and 12-residue peptide sequences derived from HIV-1 p6 and EbVp40, respectively, can bind to Tsg101, we predicted that fusion of these sequences to Gag in pENX would restore infectious particle production in the *trans*-complementation assay. Indeed, both pENX/hPTAP and pENX/ePTAP restored the processing of intracellular gag and the production of infectious HIV-1 particles almost as efficiently as the full-length p6 protein and more efficiently than the EIAV p9 protein (Fig. 5*c*). In contrast, a control peptide sequence, the influenza HA epitope tag, was inactive.

If the mechanism by which HIV-1 p6- and EbVp40-derived sequences mediate particle production is simply recruitment of Tsg101, we reasoned that directly fusing Tsg101 to Gag in the pENX context might complement pL⁻ and result in virion production. Remarkably, cotransfection of pENX/Tsg101 greatly enhanced particle production by pL⁻, almost as efficiently as pENX/p6, but in the complete absence of a viral L-domain (Fig. 6*a*). This phenotype was accompanied by a modest but definite enhancement of viral Gag processing with virus-producing cells. In addition, virions derived by complementation with ENX/Tsg101 contained processed Gag protein and were infectious (Fig. 6*a* and *b*). In fact, cotransfection with pENX/Tsg101 enhanced infectious particle production by pL⁻ approximately 10–15-fold. Notably, no *trans*-complementation resulted when Gag sequences were deleted from the pENX/Tsg101 construct, indicating that recruitment of Tsg101 to sites of particle assembly was required. Thus, the L-domain is dispensable for the production of HIV-1 particles if Tsg101 is recruited to the sites of particle assembly by another mechanism, in this case by fusion to a truncated HIV-1 Gag protein.

Deletion analysis in ENX/Tsg101 showed that the Tsg101 sequences that are required for pL⁻ complementation reside predominantly in the C-terminal half of the 390-residue protein. In fact, a Gag-Tsg101 fusion protein containing Tsg101 residues 1–250 (ENX/1–250) was inactive, whereas ENX/1–303 had low activity and both ENX/250–390 and ENX/157–390 had activities close to that of the full-length ENX/Tsg101 fusion protein (Fig. 6*a* and *b*). The inactivity and low activity of ENX/1–250 and ENX1–303, respectively, were not due to a lack of expression (Fig. 6*a*). Therefore, although the HIV-1 p6 and EbVp40 binding sites reside within the N-terminal E2 ubiquitin conjugase–like domain of Tsg101 (data not shown and ref. 11), these sequences are largely dispensable for the virion-budding function of Tsg101 provided that the remaining portion of the protein is recruited to the site of HIV-1 assembly.

Discussion

These studies establish that Tsg101 recruitment is required for efficient egress of HIV-1 virions and EbVp40 virus–like particles and define an essential virus–host interaction that is a possible target for intervention. Although the precise mechanism by which Tsg101 mediates viral budding remains unclear, recent findings provide some hints. Tsg101, otherwise known as Vps23, is a component of a 350-kDa complex, ESCRT-I, that is essential for the sorting of ubiquitinated proteins into the multi-vesicular body (MVB) in yeast and for endosomal targeting in mammalian cells[13–15]. The 'budding' and membrane-fusion events that lead to the formation of a vesicle within the MVB are topologically equivalent to the budding of enveloped viral particle, differing only in the contents of the 'vesicle' and in the cellular location at which they occur. A speculative, but entirely plausible, hypothesis is that viral proteins recruit the machinery that normally mediates MVB formation to sites of virus budding at the plasma membrane. Consistent with this notion, EbVp40 induces relocalization of Tsg101 to the plasma membrane. The finding that ESCRT-I associates with ubiquitin and with mono-ubiquitinated proteins[15] also strongly suggests a link between ubiquitin ligase recruitment by PPXY-containing retroviral late domains[9,12] and particle budding mediated by Tsg101/ESCRT-I. This finding

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may also help to explain the fact that the depletion of free ubiquitin by proteosome inhibition recapitulates the L-domain–defective phenotype in a variety of retroviruses[9,26,27].

The PT/SAP motif occurs in proteins encoded by a variety of other viruses[9], and it is possible that Tsg101 or associated proteins play a general role in viral-particle budding. It is unclear at present, however, whether the PTAP–Tsg101 interactions documented here are analogous to those in host-cell protein–Tsg101 complexes or, instead, represent a mechanism used exclusively by viruses to recruit Tsg101. Notably, the short peptide sequence employed by both HIV-1 Gag and EbVp40 to bind Tsg101 might be mimicked by small-molecule inhibitors. Such agents could have considerable antiviral activity against two lethal human pathogens of proven and potential global importance.

Note added in proof: While this manuscript was in review, Garrus *et al.*[31] reported that HIV-1 p6 binds directly to the N-terminal domain of Tsg101 and this interaction requires an intact PTAP motif. This report also showed that depletion of Tsg101 from HIV-1 producing cells results in a budding defect that recapitulates that of an L-domain–defective virus. The findings of Garrus *et al.* are entirely consistent with those described herein.

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Methods

Plasmid construction and mutagenesis. All HIV-1 proviral plasmids were based on pNL/HXB (ref. 28). To facilitate mutagenesis, an existing *Xho*I site in the *nef* gene was deleted and a new *Xho*I site was generated underlying codons 5 and 6 of p6. This generates a single amino-acid substitution within the NL/HXB p6 protein (P5L), which occurs in 10–15% of all HIV-1 isolates and does not affect virion production. This construct, which we named pWT, was used to derive the p6 series of mutants (M1–M7, Fig. 1) as well as HIV/p6/ePTAP (Fig. 4). A proviral plasmid containing two substitutions in the HIV-1 PTAP motif (P7L and P10L), referred to as pL⁻, was derived from pNL/HXB; this differs from the other p6 mutants in that it contains an intact *nef* gene. A membrane binding–defective HIV-1 construct (pG2A) was generated by introducing a single amino-acid substitution at the Gag myristoylation site in pNL/HXB. Sequences encoding the globular head of the HIV-1 matrix protein (amino-acid residues 10–110) were deleted from pNL/HXB and replaced with a *Not*I restriction site to generate pdGH, and a *Bss*HII–*Apa*I fragment encoding the dGH Gag protein was transferred to pL⁻, generating pdGH/L⁻. Two copies of an oligonucleotide encoding residues 5–16 of EbVp40 (ILPTAPPEYMEA), referred to as ePTAP, were inserted into the *Not*I site in pdGH/L⁻. Alternatively, two copies of an oligonucleotide encoding the influenza HA tag were inserted into pdGH and pdGH/L⁻.

The *trans*-complementing plasmid pENX was derived from pWT: p6, Pol, Vif and Vpr encoding sequences were excised using *Xho*I and *Sal*I and replaced with a synthetic oligonucleotide containing *Eco*RI, *Not*I and *Xho*I restriction sites. Thus, pENX-derived plasmids that express HIV-1 p6, p6(L⁻) EIAVp9, hPTAP (LEPTAPPEES), ePTAP, the HA tag or Tsg101, fused to the C terminus of a p6-deleted Gag protein, were generated by insertion of PCR products or synthetic oligonucleotides.

A cDNA encoding the EbVp40 protein was inserted into pCR3.1, which had been modified to include a HA or myc tag at the N terminus of the expressed protein. A mutation substituting a single residue within the PTAP motif (P7L) was then introduced in both cases.

Plasmids that express wild-type or mutant viral proteins in yeast, fused to a VP16 activation domain and an HA tag, were derived by insertion of PCR products into pVP16/HA (ref. 29). Alternatively, sequences encoding hPTAP or ePTAP were inserted as synthetic oligonucleotides. A plasmid that expresses a GAL4–Tsg101 fusion protein in yeast was derived by insertion of Tsg101 encoding sequences (amplified from Jurkat cDNA) into pGBKT7 (Clontech, Palo Alto, California).

Yeast two-hybrid assays. Yeast cells (Y190) were transformed with the pGBKT7- and pVP16/HA-derived plasmids described above. Transformant selection and β-galactosidase (β-gal) assays were done as previously described[29,30]. β-gal activities are expressed as absorbance units ($A_{540\ nm}$); the background activity in these assays (using pGBKT7 and pVP16/HA transformants) is approximately 0.01 absorbance units.

HIV-1 and EbVp40 particle-formation assays. HeLa or 293T cells were transfected with 2 μg of HIV-1 proviral DNA or EbVp40 expression vectors using Lipofectamine 2000 or Lipofectamine Plus (Invitrogen, Carlsbad, California). For *trans*-complementation assays, HeLa cells were transfected with 1.6 μg of pL⁻ along with 0.4 μg of pG2A or a pENX-derived plasmid. Culture supernatants, collected 48 h after transfection, were clarified by low-speed centrifugation, and particles present in 1 ml were obtained by centrifugation through a 20% sucrose cushion at 100,000*g* for 1.5 h. Viral protein content in cell and particle lysates was analyzed by HIV-1 p24 ELISA and/or western blotting.

Western-blot analysis. Yeast and mammalian cell extracts, as well as HIV-1 virion and EbVp40 particle lysates, were separated on 10% or 12% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were sequentially probed with monoclonal antibodies 183-H12-5C (HIV-1 p24), 12CA5 (HA tag) or 9E10 (myc tag) and with a peroxidase-conjugated antibody against mouse IgG, and developed using chemiluminescent substrate reagents (Pierce, Rockford, Illinois).

Infectivity assays. P4/R5 cells, which express CD4 and contain an integrated HIV-1 LTR-lacZ reporter construct, were infected with 200 μl of filtered supernatant, harvested from transfected HeLa cells. Subsequently, β-gal activities in cell lysates were measured using chemiluminescent detection reagents (galacto-Star, Tropix, Foster City, California) 48 h after infection.

Immunofluorescence. Cells (293T) were transfected with pCR3.1/myc-Tsg101, along with either an empty vector or wild-type or P7L mutant forms of pCR3.1/HA-EbVp40. Cells were transferred to coverslips 24 h after transfection and fixed the next day with paraformaldehyde. Tsg101 and EbVp40 localization was revealed using the following antibodies: as primary antibodies, 9E10 and rabbit antibodies against HA; as secondary antibodies, Alexafluor 594–conjugated antibodies against mouse IgG (Molecular Probes, Eugene, Oregon) and fluorescein-conjugated antibodies against rabbit IgG (Vector Laboratories, Burlingame, California). Nuclei were stained with Hoechst 33258. Thirty-two images at 200-nm intervals in the *z* plane were collected and deconvolved using a Deltavision microscope and software (Applied Precision, Issaquah, Washington).

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