HIV Gag mimics the Tsg101-recruiting activity of the human Hrs protein

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The HIV-1 Gag protein recruits the cellular factor Tsg101 to facilitate the final stages of virus budding. A conserved P(S/T)AP tetrapeptide motif within Gag (the “late domain”) binds directly to the NH2-terminal ubiquitin E2 variant (UEV) domain of Tsg101. In the cell, Tsg101 is required for biogenesis of vesicles that bud into the lumen of late endosomal compartments called multivesicular bodies (MVBs). However, the mechanism by which Tsg101 is recruited from the cytoplasm onto the endosomal membrane has not been known. Now, we report that Tsg101 binds the COOH-terminal region of the endosomal protein hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs; residues 222–777). This interaction is mediated, in part, by binding of the Tsg101 UEV domain to the HrsP(SAP)351 motif. Importantly, Hrs222–777 can recruit Tsg101 and rescue the budding of virus-like Gag particles that are missing native late domains. These observations indicate that Hrs normally functions to recruit Tsg101 to the endosomal membrane. HIV-1 Gag apparently mimics this Hrs activity, and thereby usurps Tsg101 and other components of the MVB vesicle fission machinery to facilitate viral budding.

Introduction

The HIV Gag protein orchestrates viral assembly and budding, and forms the structural shell of the immature virus (for review see Göttlinger, 2001). Even in the absence of any other viral proteins, HIV-1 Gag can form extracellular virus-like particles (VLPs) that resemble authentic HIV virions. Efficient release of HIV-1 virions and Gag VLPs from most cell types requires the presence of a “late domain” located in the COOH-terminal Gagp6 region (Göttlinger et al., 1991; Huang et al., 1995; Demirov et al., 2002b). All HIV-1 strains contain the late domain tetrapeptide motif P(S/T)AP (where the second position can be either Ser or Thr; Fig. 1), which is a docking site for the cellular protein, tumor susceptibility gene 101 (Tsg101; for review see Pornillos et al., 2002c). Tsg101 appears to facilitate viral budding by recruiting additional cellular factors that can catalyze release of the enveloped virion. In addition to the P(S/T)AP sequence found in HIV-1 Gag, distinct late domain sequences have also been identified and characterized in several other enveloped RNA viruses. The best characterized of these is the PPXY motif (where X is any amino acid), which binds the Nedd4 protein family of ubiquitin E3 ligases (Xiang et al., 1996; Harty et al., 1999, 2001; Strack et al., 2000; Kikonyogo et al., 2001; Yasuda et al., 2002; Timmins et al., 2003).

The P(S/T)AP late domains of HIV-1, HIV-2, and Ebola virus bind to the NH2-terminal ubiquitin E2 variant (UEV) domain of Tsg101 (Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001; Demirov et al., 2002a; Myers and Allen, 2002; Pornillos et al., 2002b; Timmins et al., 2003). UEV domains also bind ubiquitin and are structurally related to ubiquitin E2–conjugating enzymes, but lack the active site cysteine residue required for ubiquitin transfer (Moraes et al., 2001; VanDemark et al., 2001; Pornillos et al., 2002a). Tsg101 UEV also differs from the canonical E2 enzymes in that it displays a hydrophobic groove that specifically recognizes P(S/T)AP sequences (Pornillos et al., 2002a). All four P(S/T)AP residues are required for late domain activity

Abbreviations used in this paper: ESCRT, endosomal sorting complex required for transport; Hrs, hepatocyte growth factor–regulated tyrosine kinase substrate; MVB, multivesicular body; Tsg101, tumor susceptibility gene 101; UEV, ubiquitin E2 variant; UIM, ubiquitin-interacting motif; VLP, virus-like particle; Vps, vacuolar protein sorting.
Pelchen-Matthews et al., 2003). In Hrs, the FYVE domain some cell lines (Göttlinger, 2001; Raposo et al., 2002; N-myristoylated MA domain binds membranes, and con- proximal membrane-targeting domains. In HIV-1 Gag, the similarities (Fig. 1). First, both proteins contain amino- the Hrs and HIV-1 Gag proteins exhibit several intriguing et al., 2002; Bache et al., 2003b). Tsg101; Bilodeau et al., 2002; Bishop et al., 2002; Raiborg and Stenmark, 2002). Hrs is an attractive candidate for the (2) hepatocyte growth factor–regulated tyrosine kinase sub- motifs (Garrus et al., 2001), including (1) Tsg101 itself; and (2) it helps to recruit the downstream ESCRT-II, ESCRT-III, Vps4, and other proteins and complexes required to complete pro-tein sorting and vesicle formation (Babst et al., 2002a,b). Thus, recruitment of Tsg101 to the membrane surface repre-sents a key step in the commitment to vesicle formation. However, the mechanism by which Tsg101/ESCRT-I is re- cruited to the membrane has not been elucidated. Although interactions between Tsg101 and cellular P(S/T)AP motifs have not been described, we reasoned that such interactions might occur and play a role in the recruitment of Tsg101 to the endosomal membrane. Protein database searches revealed several Vps proteins with PTAP or PSAP UIM-containing proteins are substrates for the Nedd4 (yeast Rsp5p) family of ubiquitin ligases (Katz et al., 2002; Polo et al., 2001; Falquet, 2001; Bishop et al., 2002; Lloyd et al., 2002; Polo et al., 2002; Raiborg et al., 2002; Shih et al., 2002). Other UIM-containing proteins are substrates for the Nedd4 (yeast Rsp5p) family of ubiquitin ligases (Katz et al., 2002; Polo et al., 2002), and although it is not yet known whether Nedd4 ubiquitylates Hrs, the protein does harbor a PPEY motif. This sequence matches the consensus sites for cellular Nedd4 substrates and for PPXY viral late domains (Rotin et al., 2000; Freed, 2002; Pornillos et al., 2002c).

The preceding observations are consistent with a model in which (1) Tsg101 is normally recruited to the endosomal membrane through a direct interaction with Hrs; and (2) the HIV-1 Gag protein has evolved to mimic the Tsg101-recruiting functions of Hrs, and thereby redirect the machinery of MVB vesicle formation to sites of viral budding on the plasma membrane. Experiments described in this paper were designed to test key aspects of these models for Hrs function and viral mimicry.

Results

Tsg101 UEV binds the P(S/T)AP motifs from HIV-1 Gag, Tsg101, and Hrs

First, we tested whether the UEV domain of Tsg101 could bind to isolated P(S/T)AP motifs from HIV-1NL4-3 Gag and from the human Tsg101 and Hrs proteins. The Tsg101 UEV binding epitope is small, and the domain binds with the same affinity to a nine-amino acid peptide as to the in-
tect HIV-1 p6 protein (~25 μM; Pornillos et al., 2002b). Therefore, 10-amino acid peptides spanning the central P(S/T)AP motifs from Gag, Tsg101, and Hrs were tested for Tsg101 UEV domain binding in BIACore biosensor experiments. As expected, Tsg101 UEV exhibited concentration-dependent binding to the Gag PTAP peptide, and the equilibrium responses fit a simple 1:1 binding model with an equilibrium dissociation constant (Kd) of 21 ± 1 μM (Fig. 2). This interaction was specific, as the Tsg101 UEV domain did not bind to a control peptide in which the second Pro residue in the PTAP motif was mutated to Leu (Fig. 2).

The Tsg101 UEV domain also bound to P(S/T)AP peptides from Tsg101 and from Hrs (Fig. 2). However, the binding affinities were fourfold (Tsg101) and sevenfold (Hrs) weaker than Tsg101 UEV binding to the HIV-1 PTAP peptide. Hence, the Tsg101 UEV domain can bind the P(S/T)AP motifs of all three proteins, but sequences flanking the central P(S/T)AP tetrapeptides can modulate Tsg101 binding affinity significantly.

**Interaction of full-length Tsg101 and Hrs proteins**

The interaction of full-length Tsg101 and wild-type Hrs proteins was examined in directed yeast two-hybrid assays. Plasmids expressing a Tsg101–Gal4p binding domain fusion (Tsg101-BD) and an Hrs–Gal4p activation domain fusion (Hrs-AD) were cotransfected into the reporter yeast culture assays, the binding of full-length Hrs-AD to full-length Tsg101-BD typically stimulated β-galactosidase activity ~100-fold above background (Fig. 3, B–D). Therefore, we conclude that the full-length Tsg101 and Hrs proteins interact in the yeast two-hybrid assay.

**Requirements for Tsg101/Hrs binding**

Based on the known binding properties of the Tsg101 UEV domain, we hypothesized that Tsg101 might interact with Hrs via (1) an interaction between the Tsg101 UEV domain and the Hrs PSAP motif; and/or (2) an interaction between the Tsg101 UEV domain and a ubiquitin modification on Hrs. Tsg101 UEV binds P(S/T)AP peptides and ubiquitin at different sites, and distinct Tsg101 point mutations eliminate PTAP (M95A) or Ub (N45A) binding (Pornillos et al., 2002a). Therefore, these mutations were tested for their effects on the Tsg101/Hrs two-hybrid interaction (Fig. 3, B and C).

The ubiquitin-binding mutation in Tsg101 UEV (N45A) had no effect on the Hrs/Tsg101 interaction (Fig. 3 B). Likewise, alanine substitutions in the Hrs PPEY sequence had no significant effect on Tsg101/Hrs binding (unpublished data). These experiments indicated that the Tsg101/Hrs two-hybrid interaction was not mediated through ubiquitin.

In contrast, the PSAP-binding mutation in Tsg101 (M95A) reduced Hrs binding significantly (approximately sixfold; Fig. 3 C). In complementary experiments, alanine substitution of the Hrs residues 348PSAP351 sequence (HrsAP3AP) also reduced wild-type Tsg101 binding, albeit to a lesser extent (approximately twofold). The greater reduction in binding seen for the Tsg101 M95A mutation may reflect weak Tsg101 UEV binding to several additional PSAP-like motifs (e.g., Hrs 1–450) or the coiled-coil Pro/Gln-rich region (e.g., Hrs 222–559). Therefore, we conclude that the full-length Tsg101 and Hrs proteins interact in the yeast two-hybrid assay.
Thus, we conclude that full affinity Tsg101 binding requires the presence of two (or more) different Hrs regions: (1) the PSAP motif; and (2) a second region spanning the putative coiled-coil and at least part of the Pro/Gln-rich region.

**Hrs late domain activity**

Next, we tested whether the protein-recruiting functions of Hrs were sufficient to support the budding of VLPs from cultured human cells. Expression of HIV Gag–GFP fusion proteins in human cell lines recapitulates many aspects of viral assembly and budding (Hermida-Matsumoto and Resh, 2000), including the requirement for Tsg101 and other components of the MVB pathway (Garrus et al., 2001).

Human embryonic kidney 293T cells were transfected with HIV Gag expression constructs, and analyzed for protein expression and for VLP release 24–28 h later (Fig. 4). VLP release was analyzed by Western blotting of culture supernatants after sucrose cushion pelleting (Fig. 4, B and C; VLP). Intracellular Gag protein expression levels were analyzed in Western blots of cytoplasmic extracts (Fig. 4, B and C; Cell). As expected, the control Gag–GFP fusion protein expressed well and formed VLPs efficiently (Fig. 4 B, lane 1; Hermida-Matsumoto and Resh, 2000; Garrus et al., 2001). VLP release was dependent on the presence of a functional PTAP late domain, as mutation of the PTAP motif to LIRL (Gag<sup>PTAP</sup>–GFP; Fig. 4 B, lane 2) severely attenuated particle release (Göttlinger et al., 1991; Huang et al., 1995; Garrus et al., 2001).

To test whether the protein recruitment activities of Hrs could replace the late domain activity of the cis-acting Gag PTAP motif, the GFP polypeptide of Gag<sup>PTAP</sup>–GFP was replaced with the Hrs<sup>AN</sup> polypeptide (denoted Gag<sup>PTAP</sup>–Hrs<sup>AN</sup>; Fig. 4 A). As described in the previous section, the Hrs<sup>AN</sup> fragment is missing the NH<sub>2</sub>-terminal domains that normally target Hrs to the endosomal membrane, but contains both the 3<sup>48</sup>PSAP<sup>351</sup> motif and the Pro/Gln-rich region, and binds Tsg101 (Fig. 3 D). As shown in Fig. 4 B (top, lane 3), the Gag<sup>PTAP</sup>–Hrs<sup>AN</sup> fusion protein was efficiently released into the culture supernatant. Indeed, the late domain activity of the Hrs<sup>AN</sup> polypeptide (51% of total Gag protein released) was nearly as strong as the native Gag PTAP motif (57% of total Gag protein released). The Hrs<sup>AN</sup> construct also rescued particle release when fused to a Gag protein that was missing the entire p6 domain (Gag<sup>Δp6</sup>, unpublished data and see below).

Analogous results were obtained in experiments designed to test whether Gag–Hrs fusion proteins could rescue the budding of defective Gag constructs in trans (Fig. 4 C). This
wild-type Gag–GFP could rescue the release of Gag\textsuperscript{p6} when the two proteins were coexpressed in the same cell (Fig. 4 C, top, compare lane 1 and lane 2), and that rescue required the Gag–GFP PTAP sequence (Fig. 4 C, top, compare lane 2 and 3). Gag\textsuperscript{p6} release was also efficiently rescued in trans by coexpression with Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN}} (Fig. 4 C, top, lane 4). Hence, we conclude that the Hrs\textsuperscript{AN} polypeptide has potent late domain activity and can support HIV-1 Gag budding both in cis and in trans.

In addition to rescuing VLP formation in trans, some Gag fusion proteins with late domain activities can also rescue the infectivity of HIV-1 proviral constructs with budding defects (Martin-Serrano et al., 2001). For example, the wild-type Gag–GFP protein can rescue both the budding and infectivity of HIV-1 proviral constructs that harbor mutations in the PTAP-coding region of the Gag gene. However, the Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN}} construct did not efficiently rescue infectivity of the R9\textsuperscript{PTAP} proviral construct in a single cycle replication assay (unpublished data), and we speculate that this may reflect alterations in particle morphology caused by incorporation of the Hrs\textsubscript{222–777} polypeptide (see below).

Sequence requirements for Hrs late domain activity

To test the sequence requirements for Hrs late domain activity, Hrs\textsuperscript{AN} constructs missing either the PSAP motif (Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN–PSAP}}) or the Pro/Gln-rich region (Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN–Pro/Gln}}) were also tested in the two VLP release assays. As shown in Fig. 4 B, Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN}} was not released from cells (Fig. 4 B, lane 5), whereas Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN–PSAP}} (PTAP–Hrs\textsuperscript{AN–Pro/Gln}) was released (Fig. 4 B, lane 4), but with reduced efficiency as compared with the Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN}} control (27 vs. 51% total Gag protein released). Similarly, Gag\textsuperscript{p6} release was not efficiently rescued in trans by the Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN}} protein (Fig. 4 C, lane 6), although in some repetitions of this experiment, Gag\textsuperscript{p6} was released at low levels (unpublished data). The low levels of release, when seen, presumably reflected weak residual late domain activity of the PSAP sequence within the Hrs\textsuperscript{AN} polypeptide. The Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN–PSAP}} protein again rescued the release of Gag\textsuperscript{p6} to some degree (Fig. 4 C, lane 5), but was again less efficient than Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN}} (Fig. 4 C, compare lane 4 and lane 5). Also, we tested the release of a Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN}} construct with a mutation in the UIM (265LA266 → AL). This mutation abolishes Hrs ubiquitin binding and ubiquitylation of Hrs itself (Polo et al., 2002), but did not diminish VLP release. Indeed, the 265LA266 → AL mutation actually appeared to enhance Gag\textsuperscript{p6} release slightly (unpublished data). Overall, we conclude that full Hrs late domain activity requires the Hrs\textsubscript{222–777} element and COOH-terminal coiled-coil Pro/Gln-rich region, but not the UIM. These requirements correlate well with the requirements for Tsg101 binding.

EM analyses of VLPs

Transmission EM was used to confirm that the Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN}} protein was released in the form of VLPs and to examine the phenotypes of the Gag constructs that failed to bud efficiently. In control experiments, Gag–GFP VLPs appeared as enveloped, spherical particles that generally resembled immature HIV virions in both appearance and size.

Figure 4. Hrs can substitute for late domain functions of HIV-1 Gag\textsuperscript{p6}. (A) Summary of the Gag and Gag–Hrs fusion constructs and their VLP-budding phenotypes. (B) The Hrs\textsuperscript{AN} polypeptide rescues the budding arrest caused by mutation of the Gag PTAP late domain. Top, Western blot analysis of VLP release by the Gag–GFP and Gag–Hrs proteins. Bottom, Western blot showing cytoplasmic expression of the Gag–GFP and Gag–Hrs fusion proteins. 293T cells were transfected with 0.5 µg plasmid encoding Gag–GFP (lane 1), Gag\textsuperscript{p6–GFP} (lane 2), Gag\textsuperscript{PTAP–GFP} (lane 3), Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN–PSAP}} (lane 4), Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN–Pro/Gln}} (lane 5), or empty vector (lane 6). (C) Trans complementation of deficient Gag\textsuperscript{p6} budding. Top, Western blot analyzing VLP release. Bottom, Western blot showing cytoplasmic expression of the different Gag constructs. Cells were cotransfected with 1.5 µg plasmid DNA encoding Gag\textsuperscript{p6} and 0.5 µg empty vector (lane 1), Gag–GFP (lane 2), Gag\textsuperscript{PTAP–GFP} (lane 3), Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN–PSAP}} (lane 4), Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN–Pro/Gln}} (lane 5), or Gag\textsuperscript{PTAP–Hrs\textsuperscript{AN–Pro/Gln}} (lane 6).
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(100–200 nm in diameter; Fig. 5 A). However, the GFP polypeptide appeared to create discontinuities in the Gag–GFP layer, in contrast to the evenly distributed ring of Gag density that is normally observed beneath the membranes of immature HIV-1 virions and Gag VLPs.

As shown in Fig. 5 B, the Gag\textsuperscript{\text{APTAP–Hrs\textsuperscript{AN}}} protein was also released from cells in the form of enveloped VLPs that resembled authentic immature virions except that (1) the electron-dense Gag layer beneath the VLP membrane often appeared discontinuous, suggesting that the COOH-terminal GFP and Hrs polypeptides perturbed the underlying Gag lattice; and (2) VLPs formed by Gag\textsuperscript{\text{APTAP–Hrs\textsuperscript{AN}}} exhibited greater size variation and were, on average, somewhat larger than authentic immature virions. (C–E) Transmission electron micrographs of thin-sectioned 293T cells transfected with plasmids expressing Gag\textsuperscript{\text{APTAP–GFP}} (C), Gag\textsuperscript{\text{APTAP–Hrs\textsuperscript{AN}}} (D), and Gag\textsuperscript{\text{APTAP–Hrs\textsuperscript{ANPSAP}}} (E). The top images show clusters of particles associated with the cellular surface, and the bottom images show examples of classic “late domain” phenotypes in which the assembled particles remained tethered to the plasma membrane (or to one another). Bars, 100 nm.
The late domain activity of Hrs is dependent on Tsg101

Next, we examined whether the late domain activity of the Hrs\textsuperscript{AN} polypeptide required the presence of Tsg101. Cellular Tsg101 can be efficiently depleted using RNA interference (Garrus et al., 2001; Fig. 6, bottom, compare lane 1 and lane 2). As shown in Fig. 6, release of Gag\textsuperscript{Apt}–Hrs\textsuperscript{AN} VLPs from 293T cells was blocked when Tsg101 was depleted from the producer cells (Fig. 6, top, compare lane 1 and lane 2). VLP release was restored when an exogenous RNA interference–resistant Tsg101–FLAG protein (denoted Tsg101\textsuperscript{*}) was expressed (Fig. 6, compare lane 2 and lane 3). Therefore, this experiment confirmed that the late domain activity of Hrs\textsuperscript{AN} is dependent on Tsg101.

As mentioned earlier in this paper, mutation of Tsg101 residue Met95 to Ala blocks the P(S/T)AP-binding activity of the UEV domain (Pornillos et al., 2002a). Interestingly, the Tsg101\textsuperscript{*} M95A mutation also blocked the release of the Gag–Hrs\textsuperscript{AN} protein (Fig. 6, lane 4). This absolute block was somewhat unexpected because the Tsg101 M95A protein could still bind Hrs (Fig. 3 C) and because the Gag\textsuperscript{Apt}–Hrs\textsuperscript{ANPSAP} mutant protein was released from cells (Fig. 4 B). However, Hrs binding was diminished by the Tsg101 M95A mutation, and VLP release was reduced by the Hrs\textsuperscript{ANPSAP} mutation, and it is therefore possible that the reduction in Hrs binding caused by the Tsg101 M95A mutation is sufficient to block VLP budding conditions where Tsg101\textsuperscript{*} levels are limiting. Alternatively, simply recruiting Tsg101 to the site of budding may not be sufficient to support virus budding because the NH\textsubscript{2}-terminal domain of Tsg101 UEV may perform additional functions that are inhibited by the M95A mutation. In either case, the result again emphasizes the functional significance of P(S/T)AP binding by the UEV domain of Tsg101.

Discussion

Implications for cellular Hrs function

The molecular events underlying MVB biogenesis are of interest because this pathway mediates a number of important biological processes including development (Kramer, 2002), receptor down-regulation (Katzmann et al., 2002; Sorkin and Von Zastrow, 2002), and MHC-II presentation (Katzmann et al., 2002), and receptor down-regulation (Katzmann et al., 2002; Raiborg et al., 2003); (2) cooperative binding of Tsg101 to Hrs and other endosomal membrane protein(s) (e.g., ubiquitylated protein cargos); and/or (3) membrane-dependent Hrs protein release assay provides an effective functional test, in mammalian cells, for the vesicle fission activities required for both MVB biogenesis and virus budding.

Sequential recruitment of proteins onto the endosomal membrane

An intriguing aspect of MVB biogenesis is that a series of soluble complexes must be sequentially recruited from the cytoplasm onto subdomains of the endosomal membrane where ubiquitylated protein cargos accumulate and are eventually sorted into MVB vesicles. Hrs is the first of the soluble MVB factors to bind, and its recruitment is mediated, at least in part, by FYVE domain binding to PI(3)P molecules displayed on the endosomal membrane (Odorizzi et al., 1998; Urbe et al., 2000; Raiborg et al., 2001b; Katzmann et al., 2003). Hrs localization may also be cargo-dependent (e.g., through interactions between the Hrs UIM motif and the ubiquitin modifications on cargo proteins; Raiborg et al., 2002). Finally, interactions with other endosomal proteins may also play important roles in Hrs localization (Urbe et al., 2000; Raiborg et al., 2001b; Bache et al., 2002).

On the membrane, Hrs is an essential subunit of a multi-protein complex that includes Hrs, ubiquitylated protein cargos, STAM1, STAM2, Eps15, and clathrin (Asao et al., 1997; Raiborg et al., 2002; Yamada et al., 2002; Bache et al., 2003b). This complex next recruits the soluble Tsg101/ESCRT-I complex, and our experiments indicate that this occurs, at least in part, through a direct binding interaction between Hrs and Tsg101. The nature of the “switch” that allows Tsg101 recruitment only after Hrs is bound to the membrane is still unclear, but plausible possibilities include (1) avidity effects (as multiple copies of Hrs assemble at the membrane, and Tsg101 can also oligomerize; Martin-Serrano et al., 2003); (2) cooperative binding of Tsg101 to Hrs and other endosomal membrane protein(s) (e.g., ubiquitylated protein cargos); and/or (3) membrane-dependent Hrs binding activity of Tsg101.
conformational changes or phosphorylation events (Urbe et al., 2000; Bache et al., 2002).

In the next stages of MVB biogenesis, the soluble ESCRT-II and -III proteins are recruited from the cytoplasm onto the membrane (Babst et al., 2002b). Again, it seems likely that the membrane-bound ESCRT-I must be “activated” in some fashion to allow the recruitment of these downstream factors only after ESCRT-I is bound to the membrane.

Possible mechanisms of Tsg101 recruitment and activation

Unexpectedly, our binding experiments suggested that full affinity Tsg101 binding requires at least two Hrs elements, a first which involves the Hrs PSAP sequence binding to the Tsg101 UEV domain, and a second that includes downstream Hrs elements that spans the putative coiled-coil region and at least part of the Pro/Gln-rich region. Both interactions were functionally important, although mutations that disrupted the Tsg101 UEV/PSAP interaction did not completely eliminate complex formation and VLP budding in all contexts, indicating that the downstream Hrs elements may play the dominant role in Tsg101 recruitment.

The use of multiple contact sites could simply serve to increase the affinity and specificity of the Hrs/Tsg101 interaction. However, an attractive alternative model is that the downstream Hrs site serves primarily to recruit Tsg101 to sites of vesicle budding, whereas the Hrs PSAP element serves primarily as the switch that “activates” Tsg101 for ESCRT-II and/or MVB cargo binding (or for other essential events in MVB biogenesis). In this model, we envision that the soluble cytoplasmic ESCRT-I protein may exist primarily in an autoinhibited conformation in which the Tsg101 UEV domain binds its own PTAP element (i.e., in cis; Fig. 7 A). Hrs binding could then drive a conformational change in which the Tsg101 UEV domain switches to bind the PSAP element of Hrs (i.e., in trans). This activation event might also involve the ubiquitin-binding site on the UEV domain interacting with ubiquitin modifications on protein cargos, which would provide a cooperative binding mechanism for insuring that activation occurs only in the presence of both Hrs and ubiquitylated protein cargos. Although this model remains to be tested rigorously, we note that analogous autoinhibition/conformational switching mechanisms are used by other classes of proteins, such as protein kinases, to create directionality in other cellular pathways (Francis et al., 2002; Pellicena and Miller, 2002).

Viral recruitment of Tsg101

Our analyses also indicate that the structural proteins of HIV-1, HIV-2, and Ebola have evolved to mimic the normal protein-recruiting functions of the cellular Hrs protein in order to hijack the machinery that normally catalyzes MVB vesicle formation and budding (Fig. 7 B). The higher affinity P(S/T)AP element of HIV-1 Gag apparently allows the virus to compete effectively for Tsg101 binding against the lower affinity Hrs PSAP sequence. Viruses and cells may also interact differently with Tsg101 because viruses have presumably evolved to exit cells as efficiently as possible, whereas cells must carefully regulate the flux of cargo into MVB vesicles. This raises the intriguing possibility that by binding tightly to the PTAP-binding site in the UEV domain, the virus may simultaneously recruit Tsg101 and effect a conformational change that activates the protein to perform subsequent steps necessary for vesicle formation and membrane fission.

Materials and methods

Plasmid constructs

A Tsg101 mammalian expression vector was constructed by fusing the Flag epitope to the 3′ end of the full-length Tsg101 gene using PCR amplification with a downstream primer encoding the Flag sequence. The amplified product was cloned into pIRES2-EGFP (CLONTECH Laboratories, Inc.) to give pIRES-TsgFlag (Garrus et al., 2001). Seven silent mutations that render pIRES-TsgFlag resistant to siRNA (denoted pIRES-Tsg*Flag) were in-
introduced into the wild-type Tsg101-coding region \(413\text{AA}^{\text{TCTTCTCTGTCAG}}\) (mutated nucleotides are underlined) using Kunkel mutagenesis (Garrus et al., 2001). N45A and M95A mutations were also introduced into pRES-Tsg\(^*\text{Flag}\) using Kunkel mutagenesis.

All Gag and Gag-Hrs constructs were based on pGag-GFP, which contains the Rev-independent HIV-1 HBX2 Gag sequence fused to EGFP (Herzog et al., 2001). These experiments were repeated a total of six times.

The constructs were created by replacing the last three amino acids of Gag and the GFP-coding regions with either a stop codon or the appropriate Hrs sequences. Hrs constructs were PCR amplified from an EST (GenBank/EMBL/DDBJ accession no. BE276844; from American Type Culture Collection, Manassas, VA).

For yeast two-hybrid assays, Tsg101 constructs were PCR amplified from pRES-Tsg\text{Flag} and subcloned into the plasmid MP30 (Myriad Genetics), which encodes the Gal4p DNA-binding domain (BD). Hrs constructs were subcloned into the plasmid MP29 (Myriad Genetics). The resulting plasmids encoded in-frame fusions of Tsg101 with the Gal4p BD and of Hrs with the Gal4p activation domain (AD). All constructs were verified by DNA sequencing. Full cloning details are available from the authors.

**Biosensor binding assays**

BIAcore biosensor measurements of Tsg101 UEV binding to immobilized GST–peptide fusion constructs were performed as described previously (Jenkins et al., 2001; Pornillos et al., 2002a). GST–peptide fusion constructs were obtained as described previously (Elbashir et al., 2001; Garrus et al., 2001). Yeast one-hybrid assays were performed as described previously (Elbashir et al., 2001; Garrus et al., 2001). In brief, plasmid DNA encoding Gag-GFP and Gag–Hrs fusion proteins was transfected into 293T cells. Supernatants and cytoplasmic lysates were harvested 24–48 h later and analyzed by Western blotting (see below).

**Tissue culture and VLP-budding assays**

Human embryonic kidney 293T cells were grown in 2 ml Dulbecco's minimum essential medium (6-well plates) and were transfected with expression plasmids as indicated in the figure legends using LipofectAMINE \(^\text{TM}\) 2000 (Invitrogen; Garrus et al., 2001). VLP-budding assays were performed essentially as described previously (Garrus et al., 2001). In brief, plasmid DNA encoding Gag-GFP and Gag–Hrs fusion proteins was transfected into 293T cells. Supernatants and cytoplasmic lysates were harvested 24–28 h later and analyzed by Western blotting (see below).

**Electron microscopy**

VLPs from 80 ml pooled culture supernatant were pelleted through 20% sucrose cushions and prepared for thin-section EM as described previously (von Schwedler et al., 1998). Cells were prepared for thin-section EM as described previously (Garrus et al., 2001). Samples were imaged on a transmission electron microscope (Tecnai-12; Philips).

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