domain is an optimized ALIX-binding site (Vincent et al., 2003). This presumably reflects the fact that EIAV contains just a single known late domain and therefore relies heavily on ALIX to bud from cells. In contrast, the weaker HIV-1 $_{36}$YPLASL$_{41}$-ALIX late-domain interaction implies that suboptimal binding must confer a selective advantage when a functional TSG101-binding site is also present. This same trend can be seen in many different SIV strains, where the absence of a TSG101-binding site appears to correlate with the presence of an optimized ALIX-binding site (and vice versa; Bibollet-Ruche et al., 2004). Hence, viral late domains are not necessarily optimized for high-affinity binding, particularly when a virus employs multiple late domains.

We also found that ALIX can efficiently support the release and infectivity of an HIV-1 construct that lacks a TSG101-binding site, provided that the cellular ALIX levels are high enough to overcome the relatively weak binding affinity. Importantly, virus release via ALIX does not require TSG101, and vice versa. Similarly, TSG101 is not required for release of a Δp6 HIV-1 chimera that buds via a fused EIAV p9^{Gag} element (Martin-Serrano et al., 2003) nor for ALIX stimulation of murine leukemia virus budding (Segura-Morales et al., 2005). These observations all indicate that although TSG101 and ALIX can bind one another and function together in the MVB pathway, they represent independent routes out of the cell. The decisions as to which late domain(s) are employed by a particular virus, how many late domains are used, and the affinity of each binding site presumably reflect a complex optimization of the different possible exit routes from relevant host cell types.

Although the precise mechanistic role of the different cellular Class E proteins in enveloped virus budding is not yet clear, there is increasing evidence that viruses may be mimicking cellular interactions that allow membrane proteins to recruit the MVB machinery and be sorted into MVB vesicles, which ultimately leads to lysosomal degradation or release within extracellular exosomes. Interestingly, both the AP-2 adaptor complex and ALIX can bind a Tyr-based motif found in the cytoplasmic tail of the transferrin receptor (TR; although the motif does not match the YPX$_n$L consensus; Geminard et al., 2004). Vidal and colleagues have argued that the AP-2 interaction directs TR endocytosis, and the ALIX interaction may then direct the TR into exosomes that are released by maturing reticulocyte cells (Geminard et al., 2004). Both AP-2 and ALIX also bind the EIAV p9^{Gag} late domain and function in virus release (Chen et al., 2005), which suggests that EIAV may bud via the same pathway that sorts cellular TRs into reticulocyte-derived exosomes.

To date, the only well-documented example in which an ALIX-like protein binds a nonviral YPX$_n$L motif is for an ALIX homolog in *Aspergillus nidulans*, PalA, which binds tandem YPXL/I tetrapeptide repeats within PacC, a protein involved in sensing and responding to pH changes (Vincent et al., 2003). The YPXL/I motifs are required for pH-dependent proteolytic activation of PacC, although the precise role of PalA in this process is not clear. Other cellular binding partners for the ALIX V domain presumably exist, but the lack of a strong consensus sequence for the YPX$_n$L-like binding motifs complicates their identification.

Other Functional Requirements

Our studies also show that one essential function of ALIX in virus budding is to recruit the CHMP4/ESCRT-III complex. It has been challenging to establish a direct role for the ESCRT-III proteins in virus budding because human cells express 11 distinct, but related, ESCRT-III subunits, and it is therefore difficult to differentiate between nonessential versus redundant functions (e.g., see Langelier et al., 2006). Our work provides the most direct demonstration to date that ESCRT-III recruitment is required for virus budding and is consistent with previous studies showing that dominant-negative ESCRT-III constructs can block virus release (Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003) and that artificial Gag fusions that recruit ESCRT-III subunits can substitute for the $_{23}$YPDL$_{26}$ late domain in supporting EIAV p9^{Gag} release (Pineda-Molina et al., 2006). Hence, there is now increasing evidence that while enveloped RNA viruses can enter into the MVB pathway via many different binding partners, they ultimately require access to the ESCRT-III and VPS4-LIP5 complexes, which appear to be the functional machinery of MVB protein sorting/vesicle formation.

The PRR of ALIX also provides essential function(s) in virus budding. While the terminal 38 residues of ALIX could play a structural role, the high Pro and Gln content of this region (34% and 24%, respectively) makes it more likely that the polypeptide is inherently unstructured and can adopt an extended conformation that serves as the docking site for other cellular proteins. We have shown that endophilin and TSG101 binding to the PRR appear to be dispensable (or redundant) for viral budding. Determining the subset of PRR interactions that are essential for virus budding will likely shed light on the mechanistic requirements for protein sorting/vesicle formation and will further reveal how ALIX can serve as an adaptable, multidomain scaffold that links retroviral Gag proteins to essential cellular budding machinery.

EXPERIMENTAL PROCEDURES

Summary of Protein Expression, Purification, and Structure Determination

For protein-interaction studies, HIV-1 p6^{Gag} and EIAV p9^{Gag} were expressed as GST fusions in *E. coli* and purified by affinity chromatography and gel filtration chromatography (following protolytic removal of GST). ALIX$_{Bro1\text{-}V}$, ALIX$_{Bro1}$, and SeMet ALIX$_V$ were similarly expressed as GST or 6×-His N-terminal fusion proteins in *E. coli* and affinity purified for binding studies and crystallization. The 6x-His tag was left on crystallized ALIX$_{Bro1}$ but removed from ALIX$_{Bro1\text{-}V}$ and ALIX$_V$. The crystallized ALIX$_{Bro1\text{-}V}$ protein had a KK$_{268,269}$YY mutation. Structures were determined by SAD (ALIX$_V$) or molecular replacement (ALIX$_{Bro1}$ and ALIX$_{Bro1\text{-}V}$) and refined as summarized in Table S1. Full details of