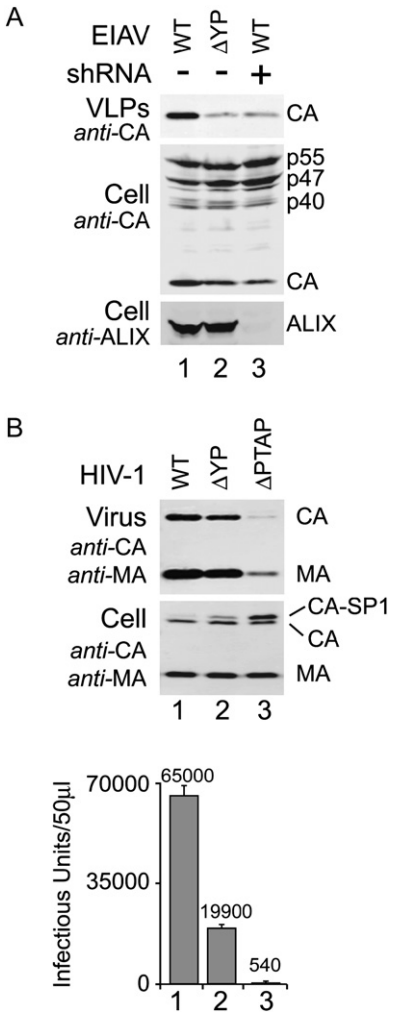

Figure 4. Requirement for ALIX in EIAV and HIV-1 Budding

(A) ALIX requirements for EIAV vector budding are shown as follows: lanes 1, wild-type (WT) EIAV vector expressed in wild-type HeLa cells (positive control); lanes 2, EIAV vector encoding a $_{23}SR_{24}$ mutation in the $_{23}YPDL_{26}$ late domain of $p9^{Gag}$ (ΔYP) expressed in wild-type HeLa cells; and lanes 3, wild-type EIAV vector expressed in cells depleted of ALIX using shRNA. Western blots are shown as follows: panel 1, virus-like particle (VLP) production (anti-CA antibody) reports the amount of successful budding and panel 2, cellular Gag protein levels (anti-CA antibody) control for expression. Note that CA corresponds to the processed central domain of Gag, p55 corresponds to full-length Gag protein, and p40 and p47 are intermediate Gag cleavage products. Cellular Gag levels were similar in all cases, although the processing was somewhat delayed in the ΔYP mutant and in the absence of ALIX. Panel 3 shows cellular ALIX levels (anti-ALIX antibody).

(B) The relative importance of ALIX and TSG101 binding for HIV-1 virus budding is shown. Lanes 1 show wild-type (WT) HIV-1 expressed in 293T cells (positive control), lanes 2 show HIV-1 encoding a $_{36}SR_{37}$ mutation in the $_{36}YPLASL_{41}$ late domain of $p6^{Gag}$ (ΔYP), and lanes 3 show wild-type HIV-1 encoding a $_{7}LIRL_{10}$ mutation in the $_{7}PTAP_{10}$ late domain of $p6^{Gag}$ (ΔPTAP). Western blots show virus production (panel 1) and cellular Gag protein levels (panel 2, anti-CA and anti-MA antibodies; MA is the processed N-terminal domain of Gag). Panel 3 shows viral titers measured in a single-cycle MAGIC assay (errors are standard deviations from three separate infectivity experiments). Note that cellular CA and MA levels were similar in all cases but that the CA-SP1-processing intermediate accumulated in the ΔYP and ΔPTAP constructs, which is a typical diagnostic of virus-budding inhibition.

release of wild-type EIAV 10-fold (compare lanes 1 and 3, upper panel). shRNA depletion of ALIX from HeLa cells was very efficient (lane 3, bottom panel), and EIAV Gag expression was not significantly affected by either the $p9^{Gag}$ ΔYP mutation or by ALIX depletion (middle panels). Gag processing was inhibited slightly, however, which is reminiscent of the Gag-processing delay observed upon inhibition of HIV-1 budding (Gottlinger et al., 1991). We therefore conclude that the $_{23}YPDL_{26}$-ALIX interaction plays a critical role in enhancing EIAV Gag release, which is in good agreement with previous studies (Martin-Serrano et al., 2003; Puffer et al., 1997; Strack et al., 2003).

The importance of ALIX for HIV-1 release was also tested by measuring the effect of the ΔYP mutation in the ALIX-binding site of HIV-1 $p6^{Gag}$. As shown in Figure 4B, this mutation reduced HIV-1 Gag release from 293T cells, as measured in both a western blot assay (compare lanes 1 and 2, panel 1) and by viral titers in a single-cycle MAGIC infectivity assay (lanes 1 and 2, panel 3). However, the reductions in release and infectivity were modest (~3-fold). In comparison, a mutation that blocked the $_{7}PTAP_{10}$-TSG101 interaction ($PTAP_{7\text{-}10}LIRL$, termed ΔPTAP) had a much more profound effect on HIV-1 release and reduced infectious titers more than 100-fold (Figure 4B, compare lanes 1 and 3). Neither late-domain mutation affected Gag protein expression, although both again delayed Gag processing, as evidenced by intracellular accumulation of the CA-SP1-processing intermediate, with the more profound effect again seen for the ΔPTAP mutation (Figure 4B, central panel). These experiments indicate that ALIX and TSG101 both enhance the release of wild-type HIV-1 from 293T cells but that the virus depends much more heavily upon the $_{7}PTAP_{10}$-TSG101 interaction. These studies again generally agree well with previous mutational analyses that utilized different HIV-1 constructs and cell types and were performed before the discovery of the $p6^{Gag}$ $_{36}YPLASL_{41}$-ALIX interaction (Demirov et al., 2002; Gottlinger et al., 1991; Huang et al., 1995).

ALIX Overexpression Rescues Release and Infectivity of HIV-1 ΔPTAP

The experiments described above demonstrate that the $p9^{Gag}$ $_{23}YPDL_{26}$-ALIX late-domain interaction plays a major role in facilitating EIAV release, yet the $p6^{Gag}$ $_{36}YPLASL_{41}$-ALIX interaction does not efficiently substitute for the $_{7}PTAP_{10}$-TSG101 interaction in supporting HIV-1 release. We hypothesized that this apparent discrepancy might reflect a reduced ability of HIV-1 to recruit ALIX owing to the lower affinity of the $p6^{Gag}$ YPX_nL-binding site. We therefore tested whether the inefficient release of the HIV-1 ΔPTAP virus from 293T cells could be "rescued" by increasing intracellular ALIX concentrations.