As shown in Figure 5, HIV-1 ΔPTAP release and infectivity were indeed stimulated very dramatically (~25-fold) by ALIX overexpression (Figure 5, panels 1 and 4, compare lanes 1 and 2). Hence, raising cellular ALIX levels could restore HIV-1 ΔPTAP infectivity to within ~20% of the wild-type virus. Importantly, ALIX overexpression did not alter intracellular Gag expression or processing (panel 2), which demonstrates that the enhancement occurred at late stages in particle assembly or budding. Control experiments also confirmed that the $p6^{Gag}{}_{36}$YPLASL$_{41}$-ALIX interaction was responsible for this enhancement because ALIX overexpression did not stimulate the release of an HIV-1 ΔPTAP virus that also lacked a functional ALIX-binding site (compare lanes 2 and 4). Indeed, the secondary ΔYP mutation actually reduced HIV-1 ΔPTAP release and infectivity by an additional 5-fold (compare lanes 3 and 4 to lane 1) presumably because the mutation also inhibited the ability of *endogenous* ALIX to mediate low-level release. These experiments demonstrate that ALIX can replace TSG101 in its role of supporting the efficient release of infectious HIV-1, provided ALIX is present at sufficient levels to overcome the modest affinity of the $_{36}$YPLASL$_{41}$-ALIX interaction.

ALIX YPX$_n$L-Binding Activity Is Required for Virus Release

The rescue of HIV-1 ΔPTAP release and infectivity upon ALIX overexpression provided a convenient assay for testing which ALIX activities were required for functional virus release. This assay was initially used to test whether ALIX mutations that inhibited YPX$_n$L binding in vitro also inhibited virus release through the ALIX pathway. As described previously, overexpression of wild-type ALIX enhanced the release and infectivity of HIV-1 ΔPTAP ~25-fold (Figure 6A, compare lanes 1 and 2). In contrast, ALIX proteins with point mutations that inhibited YPX$_n$L binding (Val498Asp, Phe676Asp, and Ile683Asp) failed to rescue HIV-1 ΔPTAP release or infectivity (compare lanes 2 and 3–5). These mutations did not affect ALIX expression or stability (panel 3), nor did they affect cellular Gag levels (panel 2). Similar data were obtained for a series of other mutations within the YPX$_n$L-binding site (Phe495Asp, Val509Asp, and Leu680Asp), whereas mutations in a second conserved hydrophobic patch located on arm1 (Leu401Asp, Ile405Asp, and Leu556Asp) did not diminish ALIX rescue significantly (<2-fold, data not shown). These data reinforce the idea that ALIX binds YPX$_n$L late domains within the site shown in Figure 2B and demonstrate that ALIX mutations that impair YPX$_n$L binding also inhibit virus release and infectivity.

ALIX Recruitment of CHMP4/ESCRT-III Is Required for HIV-1 ΔPTAP Release

In addition to binding YPX$_n$L late domains, ALIX also binds CHMP4/ESCRT-III proteins through the Bro1 domain, suggesting that ALIX may serve to connect retroviral Gag proteins to the ESCRT-III machinery (Katoh et al., 2003; Kim et al., 2005; Martin-Serrano et al., 2003; Odorizzi et al., 2003; Strack et al., 2003; von Schwedler et al., 2003). We therefore tested whether the interaction between ALIX and CHMP4A was required for virus budding. Previous work showed that mutations in an exposed hydrophobic patch on yeast Bro1p inhibited Snf7p (CHMP4) binding and prevented proper MVB sorting (Kim et al., 2005). We found that a mutation within the equivalent patch on human ALIX, Ile212Asp, similarly inhibited the ALIX$_{Bro1}$-CHMP4A interaction, as assayed in a GST pulldown experiment (Figure 6B). As expected, wild-type ALIX$_{Bro1}$ bound to a GST-CHMP4A fusion protein but not to GST alone (compare lanes 2 and 3). In contrast, the ALIX$_{Bro1,I212D}$ mutant did not bind GST-CHMP4A detectably (compare lanes 3 and 6).

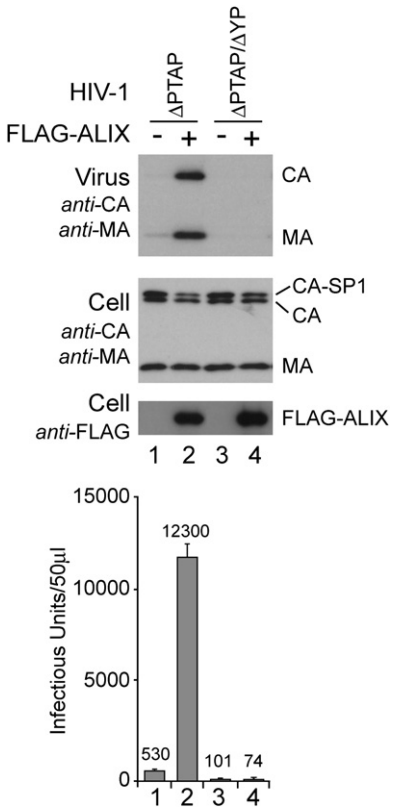

Figure 5. ALIX Overexpression Rescues HIV-1 ΔPTAP Release and Infectivity

Virus release, cellular Gag protein levels, exogenous ALIX expression, and viral infectivity are shown as analyzed by western blotting (panels 1–3) or MAGIC infectivity assays (panel 4). Lanes 1 show HIV-1 ΔPTAP cotransfected with an empty pCI-neo vector control, lanes 2 show HIV-1 ΔPTAP cotransfected with a vector expressing FLAG-ALIX, lanes 3 show HIV-1 ΔPTAP ΔYP cotransfected with an empty vector control, and lanes 4 show HIV-1 ΔPTAP ΔYP cotransfected with a vector expressing FLAG-ALIX. For reference, wild-type HIV-1 titers were typically ~65,000 IU/50 μl, and overexpression raised ALIX levels ~50-fold over endogenous protein levels as estimated by western blotting with anti-ALIX antibodies (not shown). Error bars in infectivity assays represent standard deviations of three separate infectivity experiments.