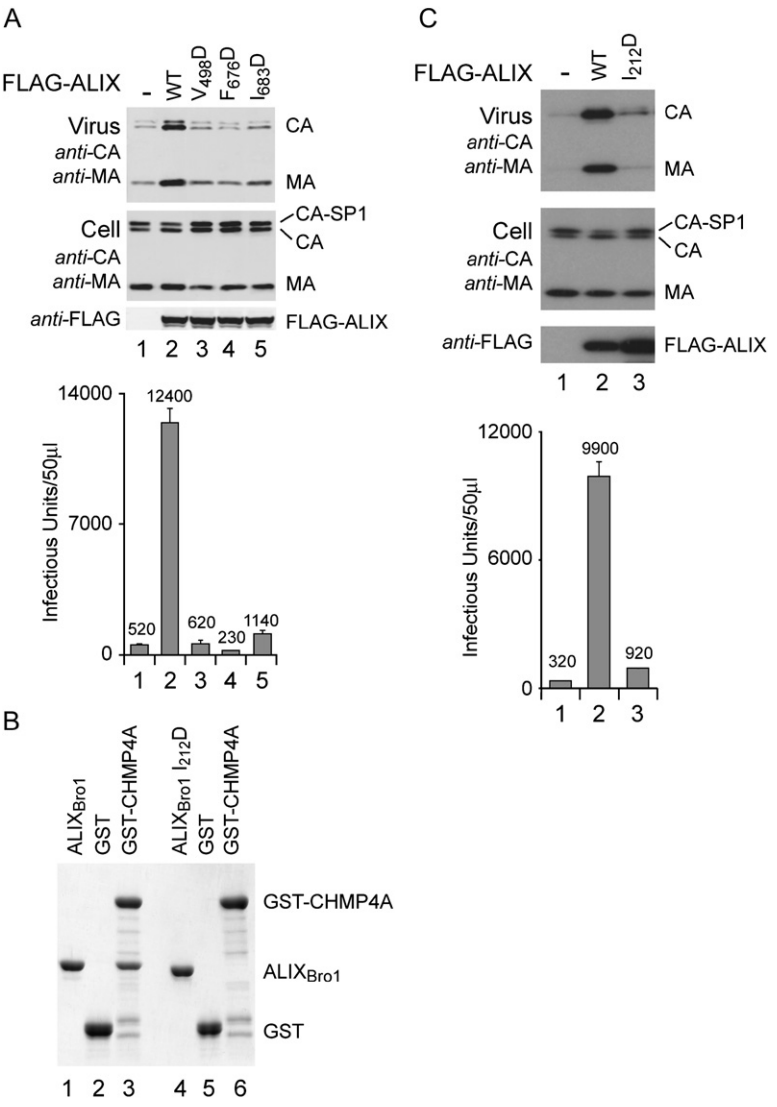

Figure 6. ALIX Mutants Lacking YPX_nL- and CHMP4-Binding Activities Do Not Support HIV-1 ΔPTAP Release and Infectivity

(A) HIV-1 ΔPTAP release and infectivity when coexpressed with an empty vector (–; lanes 1), with vectors expressing wild-type ALIX (lanes 2), or with ALIX mutants defective in YPX_nL binding (lanes 3–5) are shown. ALIX and Gag protein levels were analyzed by western blotting (panels 1–3), and infectious titers were analyzed using MAGIC assays (panel 4).

(B) This GST pulldown assay shows that the $ALIX_{Bro1}$ mutation Ile212Asp inhibits GST-CHMP4A binding. Binding experiments were performed with wild-type $ALIX_{Bro1}$ (lanes 1–3) and the $I_{212}D$ mutant (lanes 4–6). Lanes 1 and 4 show pure $ALIX_{Bro1}$ proteins (reference markers), lanes 2 and 4 show $ALIX_{Bro1}$ proteins binding to GST alone (negative controls), and lanes 3 and 6 show $ALIX_{Bro1}$ proteins binding to GST-CHMP4A.

(C) HIV-1 ΔPTAP release and infectivity are shown when coexpressed with an empty expression vector (–, lanes 1), with vectors expressing wild-type ALIX (lanes 2), or with the ALIX Ile212Asp mutant (lanes 3), which is defective in CHMP4A binding. In (A) and (C), HIV-1 ΔPTAP release, cellular Gag protein levels, exogenous ALIX expression, and viral infectivity were analyzed as described in Figure 4, with error bars representing standard deviations from three separate infectivity experiments.

We next tested whether the Ile212Asp mutation affected the ability of ALIX to rescue HIV-1 ΔPTAP infectivity. As shown in Figure 6C, the $ALIX_{I212D}$ mutant expressed well (panel 3, compare lanes 2 and 3) but failed to rescue HIV-1 ΔPTAP release or infectivity significantly (top and bottom panels, compare lanes 2 and 3). Hence, the Ile212Asp mutation strongly inhibited the ability of ALIX to support HIV-1 release, implying that one essential ALIX function in virus budding is to recruit CHMP4/ESCRT-III.

Other ALIX Functions in Virus Budding

The HIV-1 ΔPTAP rescue assay was also used to survey the functional importance of four other known ALIX properties: Tyr319 phosphorylation, TSG101 binding, endophilin binding, and interactions of the C-terminal PRR. ALIX mutants defective in each of these properties were generated and tested for their ability to rescue HIV-1 ΔPTAP release. In the first case, the ALIX Tyr319Phe mutation, which blocks phosphorylation and Src kinase binding (Schmidt et al., 2005), did not impair the rescue of HIV-1 ΔPTAP release and infectivity (Figure 7, lane 3). This result indicates that Src binding and phosphorylation do not play essential (or nonredundant) roles in ALIX virus-budding activity.

ALIX can also bind directly to the N-terminal UEV domain of TSG101 via a $_{717}PSAP_{720}$ motif located in the ALIX PRR (Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003). This interaction is of interest because it provides a potential mechanism for association of the two known late-domain-binding partners of HIV-1. As expected, mutation of the final proline in the $_{717}PSAP_{720}$ motif (Pro720Leu) eliminated TSG101 UEV binding entirely (Figure S5A). However, the $ALIX_{P720L}$ mutant fully rescued HIV-1 ΔPTAP release and infectivity (Figure 7, lane 4), which indicates that a functional TSG101-binding site was not required for ALIX to support virus budding. Indeed, we found that ALIX overexpression rescued HIV-1 ΔPTAP release even when TSG101 was