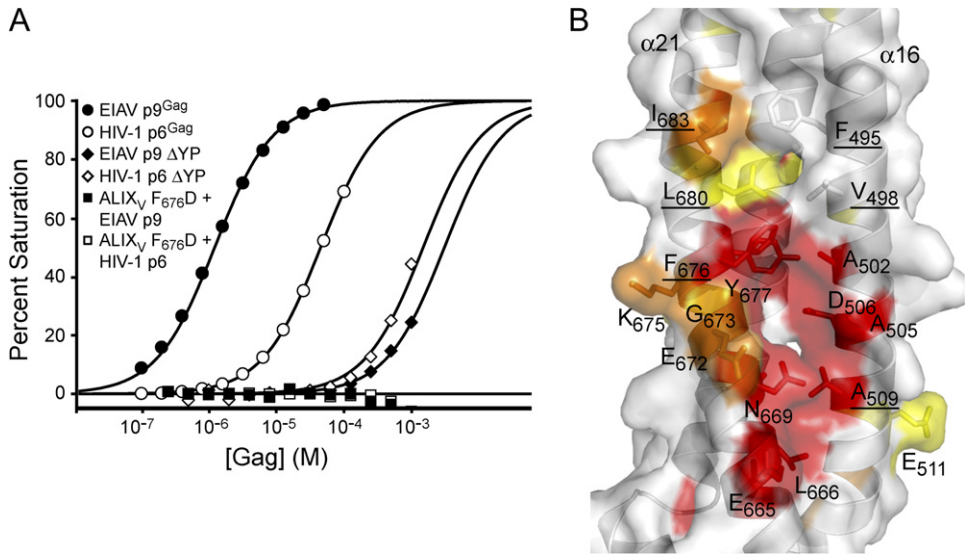


**Figure 3. Identification of the YPX$_n$L-Binding Site on ALIX$_V$**

(A) Biosensor binding isotherms for purified HIV-1 p6$^{Gag}$ and EIAV p9$^{Gag}$ constructs binding to immobilized ALIX$_V$ domain constructs are shown.

(B) YPX$_n$L-binding site on ALIX$_V$ arm2 is shown here as viewed from arm1. Residue conservation is color coded as in Figure 1E. Underlined residues were mutated and tested for YPX$_n$L-binding activity and/or for activity in the HIV-1 ΔPTAP rescue assay (see text).

positions can vary, although the biological significance of this observation remains to be elucidated.

## The ALIX V Domain Binds HIV-1 p6$^{Gag}$ and EIAV p9$^{Gag}$ YPX$_n$L Late Domains

ALIX binds and facilitates budding of retroviral Gag proteins that contain YPX$_n$L late domains, but the interaction site is not yet known. We therefore performed biosensor binding experiments to map the YPX$_n$L-binding site and measure the binding affinities of the HIV-1 p6$^{Gag}$ and EIAV p9$^{Gag}$ proteins. As shown in Figure 3A, the immobilized ALIX V domain bound both HIV-1 p6$^{Gag}$ and EIAV p9$^{Gag}$, with dissociation constants of 59 ± 15 μM and 1.2 ± 0.3 μM, respectively. In both cases, binding was specific for the YPX$_n$L late domain because YP to SR mutations (termed ΔYP; Strack et al., 2003) reduced binding affinities substantially (>15-fold). Very similar binding data were also obtained for the ALIX$_{Bro1\text{-}V}$ construct, which indicates that the Bro1 domain did not contribute to the binding interaction (Table S2).

Close inspection of the V-domain surface revealed a highly conserved hydrophobic groove on arm2 between helices 16 and 21, centered about Phe676, that was a strong candidate for the YPX$_n$L late-domain-binding site (Figures 1D, 1E, 3B, and S4). This site is exposed to solvent but is located near the base of the V, where the two arms are separated by only 5–10 Å. Phe676 sits at one end of a deep pocket (~10 Å) that is lined by a series of highly conserved residues (highlighted in red in Figure 3B). A shallower hydrophobic groove also extends ~7 Å above Phe676, although the residues that line this half of the site are less well conserved (Figures 3B, S1, and S4).

Mutational analyses confirmed that a series of conserved residues within the putative YPX$_n$L-binding site on arm2 were required for full-affinity HIV-1 p6$^{Gag}$ and EIAV p9$^{Gag}$ binding. As shown in Figure 3A, the Phe676Asp mutation abolished any detectable binding to either HIV-1 p6$^{Gag}$ or EIAV p9$^{Gag}$, which represents a >1000-fold reduction in EIAV p9$^{Gag}$ binding affinity. Mutations in two other binding-groove residues (Val498Asp and Ile683Asp) also reduced HIV-1 p6$^{Gag}$ and EIAV p9$^{Gag}$ binding affinities substantially (≥20 fold; see Table S2). These data indicate that retroviral Gag YPX$_n$L late domains bind the V domain of ALIX at a conserved site that spans the side and interior face of arm2.

## Requirements for ALIX in EIAV and HIV-1 Release

Previous studies have indicated that ALIX can function in the release of both EIAV and HIV-1 (Chen et al., 2005; Martin-Serrano et al., 2003; Strack et al., 2003; von Schwedler et al., 2003). Importantly, however, EIAV p9$^{Gag}$ contains a single known late domain (the $_{23}$YPDL$_{26}$-ALIX site), whereas HIV-1 p6$^{Gag}$ contains two late domains: a $_{7}$PTAP$_{10}$-TSG101 site and a $_{36}$YPLASL$_{41}$-ALIX site. We therefore assessed the relative importance of ALIX for the release of both EIAV and HIV-1. As shown in Figure 4A, the release of virion-associated Gag-derived CA protein from HeLa cells expressing a wild-type EIAV vector was readily detected in western blot assays (lane 1, upper panel), but the ΔYP mutation in the p9$^{Gag}$ ALIX-binding site reduced EIAV particle release 12-fold (compare lanes 1 and 2, panel 1). ALIX depletion similarly reduced the