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The HIV-1 Matrix protein does not interact directly with the protein interactive domain of AP-3 Delta

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Abstract

During the Late phase of the Human Immunodeficiency Virus Type-1 (HIV-1) replication cycle, viral Gag proteins and the intact RNA genome are trafficked to specific sub-cellular membranes where virus assembly and budding occurs. Targeting to the plasma membranes of T cells and macrophages is mediated by interactions between the N-terminal matrix (MA) domain of Gag and cellular phosphatidylinositol-4,5-bisphosphate [PI(4,5)P²] molecules. However, in macrophages and dendritic cells, a subset of Gag proteins appears to be targeted to tetraspanin enriched viral compartments, a process that appears to be mediated by MA interactions with the Delta subunit of the cellular Adaptor Protein AP-3 (AP-3δ). We cloned, overexpressed and purified the protein interactive domain of AP-3δ and probed for MA binding by NMR. Unexpectedly, no evidence of binding was observed in these in vitro experiments, even at relatively high protein concentrations (200 μM), suggesting that AP-3δ plays an alternative role in HIV-1 assembly.

Introduction

HIV-1 infects, replicates within, and eventually kills CD4⁺-bearing T-cells, macrophages, and dendritic cells. Virus assembly in T-cells occurs predominantly at the plasma membrane (PM) (18), a process initiated by the binding of a ribonucleoprotein complex comprising the viral genome and a small number of Gag proteins (24–26) to raft-like assembly sites on the PM (8, 11, 15, 28, 32, 36, 39, 40, 51, 52, 55). The targeting of Gag to the PM is mediated by phosphatidylinositol-4,5-bisphosphate [PI(4,5)P²] (37), a cellular factor that serves as a major landmark for the PM. NMR studies indicate that PI(4,5)P² can function both as a direct membrane anchor and as a trigger for myristate exposure, and the exposure of the saturated 1′-acyl chain of PI(4,5)P² and the saturated myristyl group to the membrane were proposed to serve as a mechanism for targeting the Gag:Genome complex to raft-like regions of the PM (47). PI(4,5)P² and raft-associated lipids are enriched in HIV-1 envelopes (6), consistent with this assembly mechanism, and the Gag proteins of other retroviruses appear to be targeted to assembly sites in a similar PI(4,5)P²-dependent manner (17, 46).
In macrophages and dendritic cells, the mechanisms of Gag trafficking and assembly are less well understood (1, 2, 5, 9, 13, 20, 21, 33, 34, 38, 41–43, 49, 54, 55). In these cell types, a large proportion of Gag proteins appears to be targeted predominantly to the PM or to PM-derived invaginations and internal compartments, some of which do not appear to be accessible to the extracellular milieu (23). Gag co-localizes at internal assembly sites with tetraspanins (19, 53), which appear to play roles in virus release at sites of cell-cell contact (7, 14, 16, 27, 48, 56). Recent studies indicate that Gag molecules are targeted to tetraspanin-containing assembly sites by the cellular Adapter Protein AP-3 (10, 12). APs are heterotetrameric complexes that facilitate intracellular vesicle transport by selecting vesicle cargo through interactions with signal motifs in cytoplasmic domains of transmembrane proteins, recruiting clathrin for the formation of clathrin coated vesicles (CCV) and recruiting accessory proteins for vesicle formation (31, 44, 50). AP-2 regulates receptor-mediated endocytosis at the plasma membrane and has been reported to interact with signal motifs in the cytoplasmic tail of HIV-1 gp41 thereby targeting the glycoprotein to endosomes (3, 4, 35) where it can presumably be incorporated into virions destined for the exosome pathway. AP-3 is more specifically localized to the trans-Golgi network and to peripheral endosomes, and has been shown to mediate the intracellular trafficking of CD63 to late endosomes (45) where it co-localizes strongly with Gag (33). Significantly, downregulation of the AP-3δ subunit (AP-3δ) results in attenuation of virus particle release from HIV-1 infected dendritic cells (12), and a combination of biochemical and mutagenesis studies suggested that targeting is mediated by interactions between the amino-terminal residues of the MA domain of Gag and the unstructured protein interactive domain of AP-3δ (10). To further characterize proposed interactions between MA and AP-3δ, we cloned, expressed and purified a peptide fragment corresponding to the protein interactive domain (PID, residues 641–742) of AP-3δ (AP-3δPID) for NMR-based structural studies.

Materials and Methods

DNA encoding the 107 residue protein-interactive domain of AP-3δ was subcloned into a pGEX-6P-1 (Amersham Pharmacia) vector as a C-terminal Glutathione S-transferase (GST) fusion protein. Cells were grown in LB or M9 minimal media supplemented with 99.9 % enriched 15N-ammonium chloride as the sole nitrogen (Isotec). Protein expression was induced in shake flasks with 1 mM IPTG. The cells were harvested and lysed with a microfluidizer (Micorfluidics), clarified by centrifugation, and the target protein was applied to the Glutathione Sepharose 4B matrix (Amersham). The resin was washed extensively with PBS, and an on-column cleavage step was performed with PreScission protease (Amersham) in cleavage buffer releasing the target protein from the resin. The cloning vector and subsequent cleavage resulted in the addition of 5 nonnative residues (GPLGS) to the N-terminus of the protein. Unlabeled and 15N-isotopically labeled myr(−)MA and MA were prepared as previously described (30). AP-3δPID and the Matrix proteins were exchanged into NMR buffer (50 mM sodium phosphate, pH 5.5, 5 mM DTT, and 10% D2O). NMR data were obtained with a Bruker Avance 600 MHz NMR spectrometer equipped with a cryogenic probe (protein concentrations of 50–200 μM; 35 °C).

Results

The AP-3δPID construct expressed well, was water-soluble, and exhibited the expected molecular mass (Electrospray mass spectrometry: MWobs = 13,204.1 Da; MWcalc = 13,203.7 Da). We initially titrated AP-3δPID (natural isotopic abundance) into a solution containing the 15N-labeled myr(−)MA and probed for binding by 1H-15N heteronuclear single quantum coherence (HSQC) NMR spectroscopy. As shown in Figure 1a, the AP-3δPID titrations did not lead to significant perturbations of the myr(−)MA 1H-15N HSQC spectrum. We also titrated myr(−)MA (natural isotopic abundance) into 15N-labeled
AP-3δ PID, Figure 1b. The 1H-15N HSQC spectrum of AP-3δ PID exhibited limited chemical shift dispersion consistent with an unstructured, random coil conformation, as expected. Addition of myr(−)MA did not lead to significant changes in the AP-3δ PID HSQC spectrum. These data collectively indicate that AP-3δ PID and HIV-1 myr(−)MA do not interact with each other. To determine if the lack of binding was due to the missing N-terminal myristyl group, we also titrated AP-3δ PID (natural isotopic abundance) into a solution containing the 15N-labeled myristylated MA protein. As shown in Figure 1c, AP-3δ PID did not perturb the 1H-15N HSQC spectrum of 15N-labeled myristylated MA, indicating that these proteins also do not interact.

Discussion

Considerable evidence had previously been obtained supporting a role for HIV-1 MA:AP-3 δ interactions in intracellular Gag trafficking and virus release. First, a yeast two-hybrid screen of a HeLa cDNA library using full-length Gag as bait identified the δ subunit of AP-3 as a Gag-interacting protein (10). Additional screens and truncation experiments identified residues of the protein interaction domain of AP-3 as the Gag binding site (10). In addition, directed yeast two-hybrid experiments with Gag-deletion constructs indicated that the N-terminal α-helix of the MA domain is involved in binding, and the protein interactive domain of AP-3δ was found to interact with GST-Gag fragments that contained MA (including a GST-MA fragment) (10). Finally, downregulation of AP-3δ resulted in significant reduction in virus release from HIV-1 infected dendritic cells (12) (although brefeldin A induced dissociation of AP-3 from membranes does not appear to inhibit virus production in HeLa cells or primary monocyte-derived macrophages (22)). We were therefore surprised by the current NMR results, which show definitively that neither the myristylated or unmyristylated forms of HIV-1 MA interact with the unstructured protein-interactive domain of AP-3δ. The current experiments were conducted at the relatively high protein concentrations where relatively weak interactions (Kț values of ~ 500 mM) can be readily detected. It therefore appears that the intracellular association of Gag and AP-3, and the dependence of AP-3 on virus release from dendritic cells, either do not involve interactions between MA and the protein interactive domain of AP-3δ or require other factors that are not present in our in vitro NMR assay (discussed further below).

AP-3δ is one of many cellular proteins that have been implicated in aspects of intracellular Gag trafficking and virus assembly and release (for example, see (1, 5, 8, 21)). For example, the cellular “tail interacting protein of 47 kDaltons” (TIP47) was also implicated as a MA-binding partner, based in part on a yeast two-hybrid screen (29). We recently obtained NMR evidence that the intact TIP47 molecule, which contains a membrane-binding domain, is capable of interacting with MA, but the cellular receptor-binding domain of TIP47 and a TIP47 construct that lacks the membrane-spanning domain do not bind MA (Eric Freed, M.F. Summers, and co-workers, manuscript in preparation). It appears that MA interactions with TIP47 are promoted in aqueous solution by non-native interactions with residues that would normally be sequestered within the membrane, which may explain the yeast 2-hybrid results obtained for this system. Non-native interactions in the absence of cellular constituents might also explain why MA:AP-3δ interactions were observed in the yeast 2-hybrid screens, whereas MA:AP-3δ PID binding could not be detected in solution by NMR techniques.

In summary, although there is now good evidence that HIV-1 Gag is targeted primarily to the PM or PM-derived internal membranes in macrophages and dendritic cells, the roles of AP-3 in membrane targeting and virus assembly remain unclear. Quantitative binding studies involving cellular factors, and perhaps with larger and more biologically relevant fragments of Gag, are warranted.
References


Highlights

Previously proposed AP-3:MA binding model tested by NMR.

Findings unexpectedly show that the Protein Interactive Domain of AP-3 does not bind HIV-1 MA.

Findings suggest AP-3 may play other roles in Gag trafficking and HIV-1 assembly.
Figure 1.

$^1$H-$^{15}$N HSQC NMR titration experiments showing that myristylated and unmyristylated forms of HIV-1 MA do not interact with the protein-interactive domain of AP-3δ. (a) $^{15}$N labeled myr(−)MA titrated with unlabeled AP-3δ PID; AP-3δ PID: MA ratios: 0:1 (black), 0.5:1 (yellow), 1:1 (green), and 2:1 (red). (b) $^{15}$N labeled AP-3δ PID titrated with unlabeled HIV-1 myr(−)MA; MA: AP-3δ PID ratios: 0:1 (black), 0.5:1 (yellow), 1:1 (green), and 2:1 (red). (c,d) $^{15}$N-labeled myristylated MA titrated with AP-3δ PID (natural isotopic abundance); AP-3δ PID:MA ratios: 0:1 (black), 0.5:1 (yellow), 1:1 (green), and 2:1 (red). The expansion in (d) is provided to more clearly distinguish between the highly similar spectra.