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Characterizing Cellular Factors Involved in HIV-1 Gag Trafficking to Assembly Sites

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Abstract

Background
In the late stages of the viral replication cycle, the Gag precursor, Pr55Gag, is synthesized in the cytoplasm, cotranslationally myristylated, and recruited to the plasma membrane (PM) through binding to the phospholipid $PI(4,5)P_2$. Though the mechanisms of virus release are well studied, the trajectory by which Gag traffics remains one of the least understood aspects of HIV-1 replication. We hypothesize that HIV-1 Gag requires a network of cooperative endosomal and secretory pathway cofactors to localize to sites of virus assembly. We previously determined that overexpression of the Golgi-localized γ-ear containing Arf-binding (GGA) proteins reduces HIV-1 particle production by impairing Gag trafficking to the membrane through disruption of Arf (ADP-ribosylation factor) protein activity. We therefore aim to characterize the role of the Arf family of vesicular trafficking proteins in the context of HIV-1 assembly and release.
Methods
We screened dominant negative (DN) mutants of the Arf family members to determine the effect that functional disruption of these proteins has HIV-1 release.
Results
Expression of Arf1DN potently inhibits not only HIV-1 release efficiency, but also processing of the viral envelope (Env) precursor gp160, and subsequently, particle infectivity. Interestingly, Arf3DN does not confer the same phenotype, despite greater than 96% sequence identity with Arf1. We demonstrate that Arf1DN inhibits virus release independent of matrix (MA), Env, and Nef as well as of mutants defective in budding, maturation, and membrane targeting. This virus release defect is not due to the mis-targeting of Gag to intracellular sites of assembly or the loss of Gag membrane binding, but rather due to the accumulation of Gag at intracellular membranes away from the PM. The retention of Gag membrane binding function, but the loss of assembly may be due to a defect in Gag oligomerization. Treatment of cells with Arf inhibitors also results in a defect in Env processing and virus release.
Conclusions
This study suggests that disruption of Arf1 function severely affects the early steps of HIV-1 Gag trafficking. The accumulation of Gag at internal membranes upon disruption of Arf1 suggests Gag may interact with other endosomal/secretory membranes and vesicles prior to association with the PM. The characterization of Arf1DN-mediated re-localization of Gag to specific membranes will assist in the elucidation of the steps in the HIV-1 Gag trafficking pathway.

The mechanism by which Gag is targeted to the PM is still poorly understood

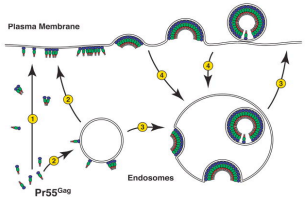


Figure 1: Potential Gag trafficking routes. From: Ono, A. *Future Virology*. 2009.

Disrupting the function of the Arf family of vesicular trafficking proteins

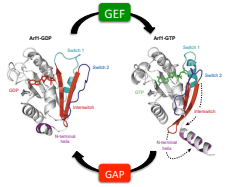


Figure 2: Arf family members reversibly associate with membranes during their GTP cycle, regulated by cellular GEF and GAP proteins. Figure modified from Gillingham, A.K, et al. *Annual Review of Cell and Developmental Biology*. 2007.

Overexpression of WT Arf proteins minimally inhibits the virus release of WT HIV-1

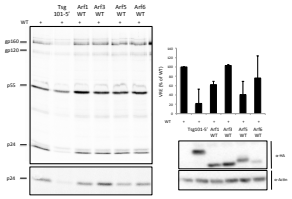
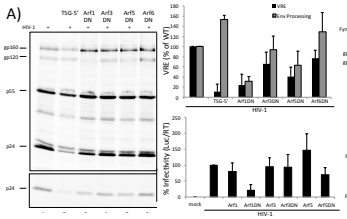


Figure 3: HeLa cells were transfected with pNL4-3 WT alone or co-transfected with the indicated expression constructs. Cells were starved for 30 minutes, 24 hours post-transfection, and then metabolically labeled with ^{35}S-Met/Cys for 3 hours. Virus was pelleted; cell and virus lysates were immunoprecipitated and run on SDS-PAGE and exposed to a PhosphorImager for densitometry analysis. VRE was calculated as the amount of virion p24 divided by total Gag (cell Pr55Gag + cell p24 + virion p24). All values were normalized relative to WT. Data from three independent experiments are shown as means ± SD.

Expression of Arf1DN results in defects in HIV-1 particle release, Env processing, and infectivity



Arf1 DN-mediated inhibition of virus release and Env processing occurs independently of HIV-1 MA

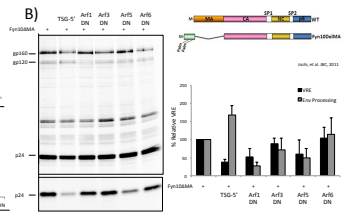


Figure 4: HeLa cells were transfected with pNL4-3 WT (A) or pNL4-3 Fyn10ΔMA (B) alone or co-transfected with the indicated expression constructs. Cell and virus lysates were processed as in Figure 3. Env processing was calculated as the ratio of cellular gp120/gp160. Virus containing supernatants were used to infect TZM-bl reporter cells and infectivity is displayed as luciferase over viral input values. All values were normalized relative to WT. Data from three independent experiments are shown as means ± SD. B) Schematic of constructs used (Joshi, A., et al. *JBC*. 2011).

Arf1 DN inhibits virus release of HIV-1 mutants defective in budding, maturation, PM targeting, and deficient in Nef and Env

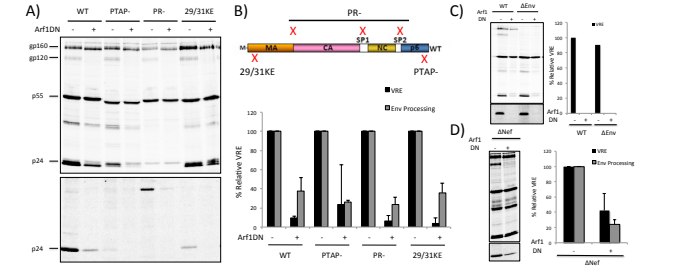


Figure 5: A) HeLa cells were transfected with pNL4-3 WT, or the indicated mutants in the presence or absence of Arf1 DN. Cell and virus lysates were processed as in Figure 3. B) Schematic of mutant viruses (modified from Fig. 4B). C) HeLa cells were transfected with pNL4-3 WT or pNL4-3 ΔEnv in the presence or absence of Arf1 DN. Cell and virus lysates were processed as above A). D). HeLa cells were transfected with pNL4-3 ΔNef and processed as above. All values normalized to viruses in the absence of Arf1 DN. . Data from three independent experiments are shown as means ± SD.

Elements of the N- and C-terminus of Arf1 DN mediate the inhibition of virus release and Env processing

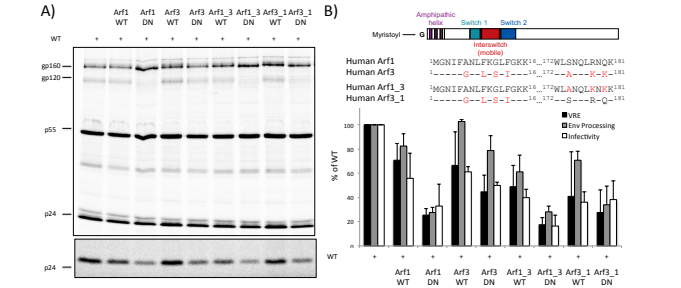


Figure 6: A) HeLa cells were transfected with pNL4-3 WT alone or co-transfected with the indicated expression constructs. Cell and virus lysates were processed as in Figure 3. Virus infectivity was measured as in Figure 4. Luciferase values are expressed normalized to viral input. All values were normalized relative to WT. Data from three independent experiments are shown as means ± SD. B) Schematic of Arf constructs used (modified from: Gillingham, A.K, et al. *Annual Review of Cell and Developmental Biology*. 2007.

Arf1 DN expression does not result in a defect in Gag membrane binding

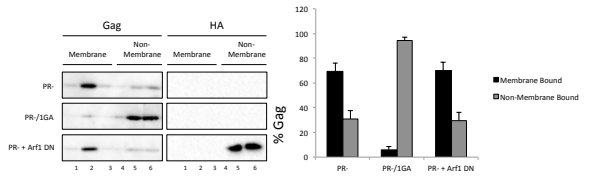


Figure 7: A) HeLa cells were transfected with pNL4-3 PR- alone or co-transfected with the indicated expression constructs. Cells were collected and post-nuclear supernatants were subjected to membrane flotation analysis. Six fractions were collected per sample post-16hr flotation ultra-centrifugation, divided into membrane-containing or non-membrane containing fractions and subjected to western blot analysis for either HIV-1 Gag protein or HA-tagged Arf proteins. Data from three independent experiments are shown as means ± SD.

Arf1 DN does not mis-target Gag to intracellular sites of assembly

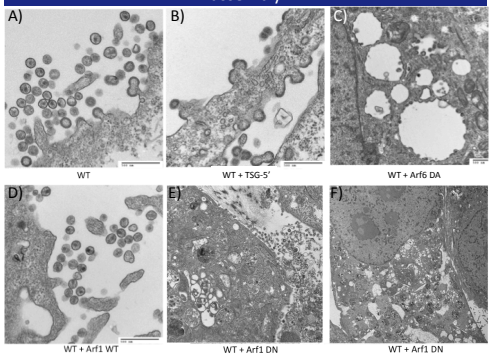


Figure 8: HeLa cells were transfected with pNL4-3 WT alone (A) or co-transfected with the indicated expression constructs, TSG-5' (B), Arf6 DA (C), Arf1 WT (D), Arf1 DN (E, F). Twenty-four hours post-transfection, cells were fixed in 2% Glutaraldehyde, 0.1M Sodium Cacodylate, and processed for EM (electron microscopy).

Arf1 DN decreases the accumulation of Gag at the plasma membrane

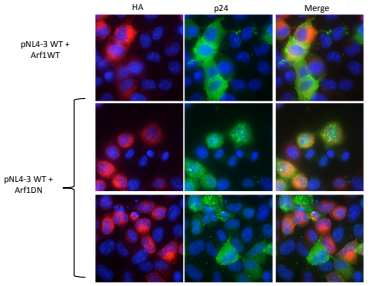


Figure 9: HeLa cells were transfected with the indicated expression constructs in the presence or absence of pNL4-3 WT. Twenty-four hours post-transfection, cells were fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton-X, blocked, and incubated with FITC-conjugated anti-p24-core antigen or anti-HA primary antibodies overnight. Cells were washed, and incubated with anti-IgG_{2a} AlexaFluor 594 secondary antibody for one hour. Cells were washed, mounted with DAPI stain, and imaged on a DeltaVision deconvolution microscope.

Arf1 DN affects the distribution of the cis-Golgi similar to the effects of Brefeldin A (BFA)

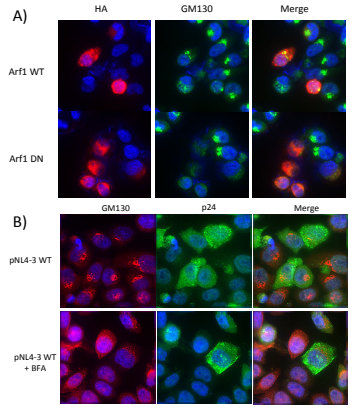


Figure 10: A) HeLa cells were transfected with the indicated expression constructs in the presence of absence of pNL4-3 WT. Twenty-four hours post-transfection, cells were processed as in Figure 9 with anti-p24-core antigen, anti-HA, or anti-GM130 primary antibodies

GBF-1 inhibitors do not inhibit HIV-1 virus release but affect Env processing

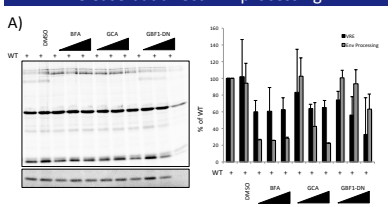


Figure 11:. HeLa cells were transfected with pNL4-3 WT alone or co-transfected with the GBF1-DN expression construct. Cell and virus lysates were then processed as in Figure 3 while labeling in the presence of BFA or GCA. Data from two independent experiments are shown as means ± SD.

Disruption of BFA-insensitive GEFs with LM11 results in defects in both HIV-1 virus release and Env processing

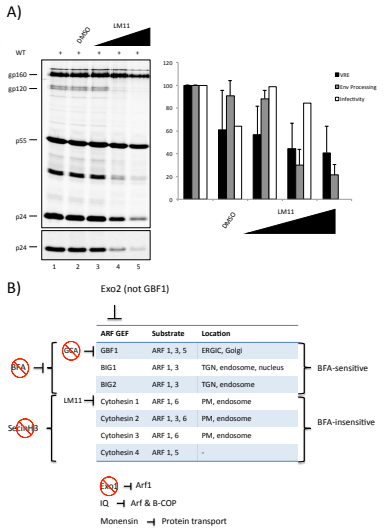


Figure 12: A) HeLa cells were transfected pNL4-3 WT. Cell and virus lysates were processed as in Figure 11. Data from four independent experiments are shown as means ± SD. B) A summary table of the major Arf GEFs, substrates, and locations within the cell. The general secretory and protein transport inhibitors as well as Arf GEF specific inhibitors tested are indicated. Figure adapted from Gillingham, *et al. Annual Review of Cell and Developmental Biology*. 2007.

Summary

- Arf1 DN disrupts endogenous Arf1 function and consequently inhibits WT HIV-1 virus release, Env processing, and infectivity.
- The inhibition of HIV-1 virus release by Arf1 DN occurs independently of HIV-1 MA, Env, and Nef
- Arf3 DN does not inhibit virus release, Env processing, or infectivity, 96% sequence identity with Arf1.
- Chimeric Arf3 DN constructs containing the N- or C-termini of Arf1 are able to inhibit virus release, Env processing, and infectivity, suggesting that both the stability and the site of membrane interactions encoded by the N- and C-termini of Arf1 are critical to the inhibitory phenotype.
- Arf1 DN is able to inhibit the virus release and Env processing of HIV-1 mutants defective in budding (PTAP-), maturation (PR-), and PM targeting (MA 29/31 KE), suggesting that the inhibition occurs in early stages of Gag trafficking.
- Arf1DN expression does not result in a defect in Gag membrane binding, suggesting that Gag may be sequestered on an internal membrane.
- The inhibition of Gag trafficking by Arf1 DN does not result in the mis-targeting of Gag to sites of intracellular assembly, suggesting instead, that this inhibition results in inefficient Gag plasma membrane binding.
- Arf1 DN expression results in the accumulation of Gag at intracellular foci, away from the PM.
- Arf1 DN affects the distribution of the cis-Golgi similar to that of BFA, however, BFA does not inhibit HIV-1 virus release efficiency.
- Targeting BFA-insensitive GEFs with inhibitors of Arf activation inhibits HIV-1 virus release and Env processing.