The HIV Antisense Protein ASP is a Transmembrane Protein of the Viral Envelope

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Introduction

The negative strands of many retroviruses contain open reading frames (ORF) expressed from the 3'LTR. The most intensely investigated retroviral antisense protein is the HTLV-1 HBZ protein. The existence of an ORF in the negative strand of the HIV-1 genome was first predicted 30 years ago. This ORF is located at the gp120/gp41 junction of the *env* gene, and encodes a highly hydrophobic protein of ~189 aa. The product of the HIV-1 antisense ORF (termed Anti-Sense Protein, or ASP) presents two putative transmembrane domains, two closely-spaced cysteine triplets, and an SH3 domain-binding motif (PxxPxxP). Several lines of evidence suggest that ASP is expressed *in vivo*. First, the ASP ORF is found only in group-M HIV-1 (responsible for the worldwide pandemic) but not groups N, O, or P. Second, preservation of the *asp* ORF in group-M HIV-1 did not occur by chance, but rather under selective pressure, suggesting a role – albeit non-essential – of ASP in viral spread. Finally, humoral and cellular immune responses against ASP are present in peripheral blood of HIV-1 infected individuals.

Here we tracked the expression and dynamics of ASP during non-productive and productive chronic infection with full-length, replication competent HIV-1 in a panel of lymphoid and myeloid cell lines using flow cytometry and microscopy techniques. We found that during non-productive infection ASP dwells in the nucleus displaying a polarized distribution, while during productive infection ASP translocates to the cytoplasm and is expressed on the plasma membrane. Moreover, after viral budding and release, ASP is incorporated in the viral envelope, thus becoming a structural protein of the HIV-1 virion. Finally, ASP-deficient viruses display reduced replication rates compared to the wild type strain. Altogether, our results suggest that ASP may play a role in HIV-1 replication and/or spread, and identify ASP as a possible new target for therapeutic and vaccine interventions.

Results

We produced a monoclonal anti-ASP antibody (324.6) against an epitope located between the two putative transmembrane domains of the protein (**Figure 1**). This antibody detected ASP expression in the nuclei of chronically infected myeloid (U1 and OM-10.1), and lymphoid (H9-IIIB, H9-CC, H9-MN, H9-RF, J1.1, ACH2 and 8E5) cell lines but not in their parental uninfected cell lines (**Figures 2**) in flow cytometry.

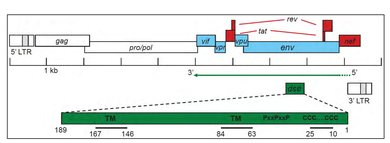


Figure 1: HIV-1 Antisense Protein, ASP (*Clerc et al., 2011*).

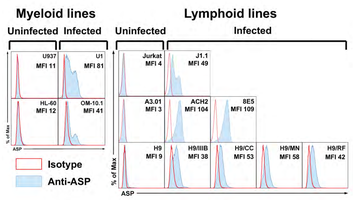


Figure 2: Nuclear expression of ASP by flow cytometry in unstimulated myeloid (left) and lymphoid cell lines (right).

Confocal microscopy analyses showed that ASP displays a polarized distribution within the nuclei of non-productively infected cells(**Figures 3**).

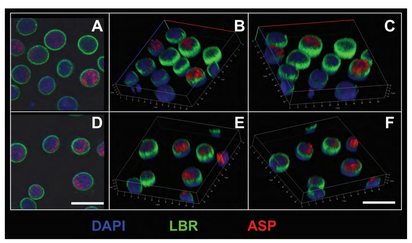


Figure 3: Sub-nuclear distribution of ASP in U1C8 cells. A and D: 2D images showing ASP (red), DAPI (blue) and Lamin B Receptor (LBR; green). B, C, E and F: 3D images reconstructed from *z*-stack images. ASP is only present in the nuclei, and shows a polarized distribution. The size bars represent 10 μm for all images.

After reactivation of HIV-1 expression with PMA in U1C8 cells, ASP translocated to the cytoplasm and co-localized with HIV-1 p24 (**Figures 4**).

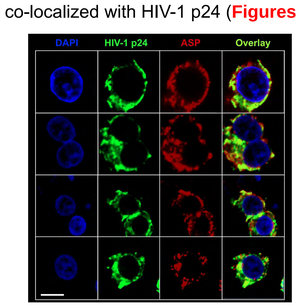


Figure 4: Co-localization of ASP and p24 in the cytoplasm of productively infected U1C8 cells. 2D images showing ASP (red), DAPI (blue) and HIV-1 p24 (green). ASP is detectable in the cytoplasm, and co-localizes with p24. The size bar shown in the lower left image represents 2 μm for all images.

ASP and HIV-1 gp120 localize on the surfaces of primary human CD4⁺ T cells and primary human monocyte-derived macrophages infected with HIV-1_{Ada-M} (**Figures 5 and 6**)

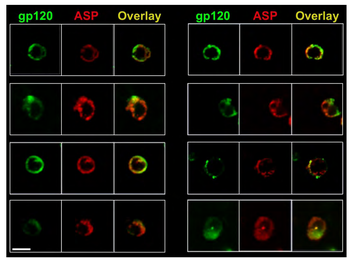


Figure 5: Co-localization of gp120 and ASP in the cell surface of primary cells (CD4⁺ T). 2D images showing ASP (red), DAPI (blue) and HIV-1 gp120 (green).

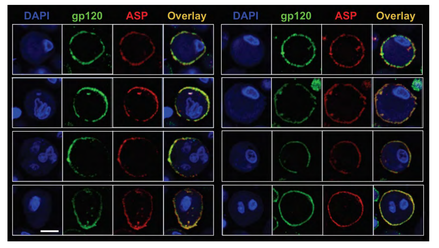


Figure 6: Co-localization of gp120 and ASP in the cell surface of primary cells (Macrophages). 2D images showing ASP (red), DAPI (blue) and HIV-1 gp120 (green).

Reactivation of HIV-1 expression with PMA in U1C8 and OM-10.1 cells also led to the expression of ASP on the plasma membrane (**Figure 7 and 8**)**.** Detection of ASP on the cell surface without membrane permeabilization indicates that the epitope detected by the anti-ASP antibody is exposed to the extracellular milieu.

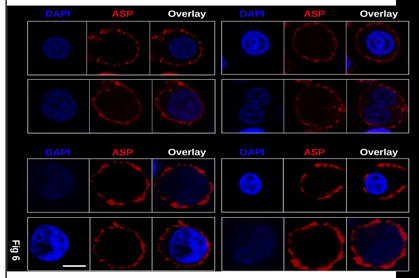


Fig 6

Figure 7: Expression of ASP (red) on the surface of productively infected U1C8 (top) and OM-10.1 (bottom) cells. The size bar shown in the lower left image represents 2 μm.

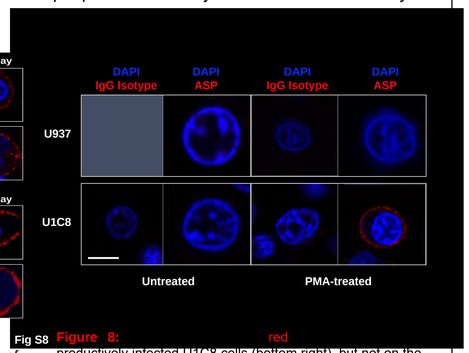


Fig S8

Figure 8: red productively infected U1C8 cells (bottom right), but not on the surface of non-productively infected U1C8 cells (bottom left), or U937 cells (top panels). The size bar shown in the lower left image represents 5 and 2 μm for the IgG and ASP panels, respectively.

ASP was detectable on the cell surface of all productively infected cell lines we analyzed (**Figure 9**). Moreover, we found that ASP and HIV-1 gp120 co-localized on the cell surface of productively infected U1C8 (Manders' Overlap Coefficient ~76%; **Figures 10**).

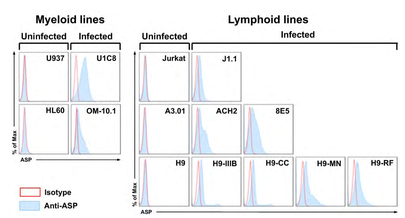
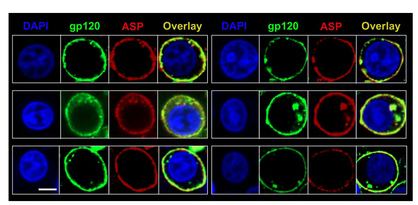
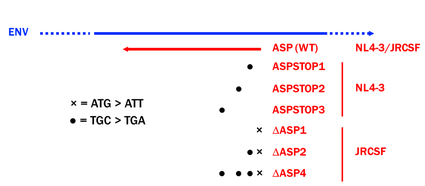


Figure 9: Cell surface expression of ASP by flow cytometry in PMA-stimulated myeloid (A) and lymphoid cell lines (B). Surface expression of ASP by flow cytometry after HIV-1 reactivation on myeloid (C) and lymphoid cell lines (D).



Figures 10: Co-localization of ASP and HIV-1 gp120 on the cell surface of productively infected U1C8 cells. 2D images showing ASP (red), DAPI (blue) and HIV-1 gp120 (green). ASP is detectable on the cell surface, and co-localizes with gp120 (Mander's Overlap Coefficient ~76%).



Figures 11: Diagram showing mutations in the ASP open reading frame:. X, indicates ATG to ATT, altering the start codon. ●, **indicates TGG to TGA a mutation that induces a stop codon**

To test whether ASP expression impacts viral replication, we mutated single nucleotides in the HIV-1 in the ASP open reading frame without affecting the opposite strand (**Figure12A**). ASP-deficient viruses compared to the wildtype virus (**Figure 12B**).

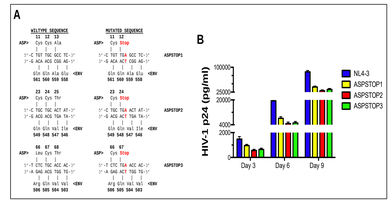


Figure 12: Impact of ASP Expression on HIV-1 Replication. A: Single nucleotides in the ASP ORF of HIV-1_{NL4-3} (left) were mutated to introduce early *stop* codons (right) without altering the amino acid sequence of Env on the opposite strand. B: HIV-1_{NL4-3} mutants (ASPSTOP1-3) replicate less efficiently than wildtype virus.

ation, we generated three JRCSF-derived mutants in o ATT. In addition, in two of the mutant clones we **Figure 11**). Again, none of these single nucleotide f Env on the opposite strand. We then used these and monocyte-derived macrophages from multiple donors. In both cases, replication of mutant clones was significantly lower compared to wild-type JRCSF strain (**Figure 13 and 14).**

Fig 10

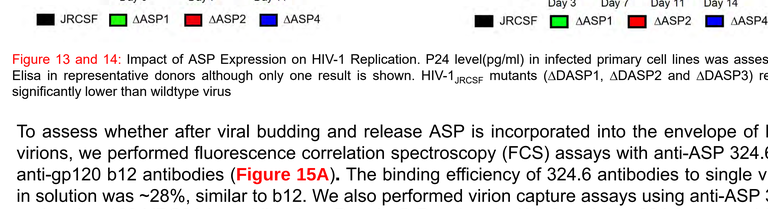


Figure 13 and 14: Impact of ASP Expression on HIV-1 Replication. P24 level(pg/ml) in infected primary cell lines was assessed by Elisa in representative donors although only one result is shown. HIV-1_{JRCSF} mutants (ΔDASP1, ΔDASP2 and ΔDASP3) replicate significantly lower than wildtype virus

To assess whether after viral budding and release ASP is incorporated into the envelope of HIV-1 virions, we performed fluorescence correlation spectroscopy (FCS) assays with anti-ASP 324.6 and anti-gp120 b12 antibodies (**Figure 15A**)**.** The binding efficiency of 324.6 antibodies to single virions in solution was ~28%, similar to b12. We also performed virion capture assays using anti-ASP 324.6 and anti-gp120 VRC01 immobilized on Protein G Dynabeads. We found that 324.6 captured HIV-1 virions with an efficiency similar to VRC01 (**Figure 15B**). Altogether, these studies show that ASP is expressed on the surface of HIV-1 particles.

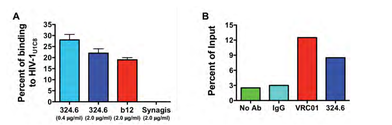


Figure 15: A. Fluorescence Correlation Spectroscopy analysis of single HIV-1 particles from U1C8 cells using anti-ASP 324.6, anti-gp120 b12 and anti-RSV Synagis antibodies. **B.** Virion capture assay of single HIV-1 particles from U1C8 cells using anti-ASP 324.6 and anti-gp120 VRC01 antibodies. Both assays indicate the presence of ASP on the surface of HIV-1 virions.

Conclusions

Our results indicate that ASP is expressed in the nuclei of non-productively infected cells, and on the cell surface of productively-infected cells where it co-localizes with gp120. ASP is also found on the envelope of viral particles, and thus is a structural protein of HIV-1. Further, ASP expression increases viral replication. ASP may represent a new target for therapeutic or preventive vaccines.

References

1. Clerc I, et al. (2011) Polarized expression of the membrane ASP protein derived from HIV-1 antisense transcription in T cells. Retrovirology 8:74.
2. Cassan E, et al (2016) Concomitant emergence of the antisense protein gene of HIV-1 and of the pandemic. Proc. Natl. Acad. Sci. USA 113:11537–11542.

Funding

NIH Grants AI120008 and AI124776 to F.R.; NIH grants AI116274 and AI129769 to M.P.; NIH grants GM118766 and GM118766-S1 to K.R.