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LETTER TO THE EDITOR

Enhancement of endocytic uptake of HIV-1 virions into CD4-negative epithelial cells by HIV-1 gp41 via its interaction with POB1

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We previously used recombinant soluble gp41 (rsgp41) as a target to screen a human bone marrow cDNA library by the yeast two-hybrid assay, and we identified an HIV-1 gp41-binding protein, human POB1 (the partner of RalBP1). We found that the gp41-binding site was located at the C-terminal region (aa462–521) of POB1 and the POB1-binding site was on the N-terminal heptad repeat (NHR) region of HIV-1 gp41.[1] However, it is unclear whether this interaction has any effect on HIV-1 entry into the host cell. Here, we showed that gp41 could co-immunoprecipitate with POB1 and co-localize with POB1 in NRK cells transfected with pEYFP-N1-rsgp41 and pECFP-N1-POB1. HIV-1 gp41 effectively promoted the internalization of epidermal growth factor (EGF), whereas both gp41 and EGF significantly enhanced the entry of HIV-1 virions into CD4-negative A431 epithelial cells. This enhancement was attenuated by the endocytosis inhibitor, chlorpromazine. These results indicate that HIV-1 gp41 promotes viral endocytosis in a CD4-independent manner by interacting with the host protein POB1, suggesting a new role for gp41 in the endocytic uptake of virions into CD4-negative epithelial cells and providing a new target for the development of therapeutics and prophylactics against HIV/AIDS.

In this study, we cloned a full-length POB1 cDNA by PCR amplification from the human brain cDNA library that was reported to have high copies of POB1 messages.[2] Sequencing of this cDNA clone revealed that it encodes a POB1-containing 460 amino acid residues (POB1-460). Compared with the wide-type POB1-521, POB1-460 has a 61 amino acid deletion and 5 amino acid substitutions. We tested the interaction between POB1-460 and rsgp41 in cells by co-immunoprecipitation assays.[3] Lysate of 293T cells that were co-transfected with pCMV-HA-rsgp4 plasmid (control: pCMV-HA plasmid) and pCMV-Flag-POB1-460 (control: pCMV-Flag) was incubated with anti-HA mAb and co-immunoprecipitated with Protein A/G beads. We then used anti-Flag mAb to detect bound proteins and found that Flag-tagged POB1-460 was co-precipitated with HA-rsgp41 (Figure 1a). Confocal microscopy was used to observe NRK cells that were co-transfected with pEYFP-N1-rsgp41 and pECFP-N1-POB1. Colocalization of dots that represent the GFP-labeled rsgp41 and YFP-labeled POB1 clearly demonstrated the colocalization of rsgp41 and POB1 (Figure 1b). These results confirmed the interaction between gp41 and POB1-460.

POB1 is involved in the process of receptor-mediated EGF internalization.[2] The A431 cell line, which was derived from a female with epidermal carcinoma and carries large numbers of EGF-binding sites, has been widely used as a model cell line for studying the regulation of EGF and signal transduction (epidermal growth factor receptor).[4] Therefore, we used this cell line to investigate whether HIV-1 gp41 affects EGF internalization. A431 cells that were transfected with pcDNA3-rsgp41-EGFP (control: pcDNA3-EGFP) plasmid were treated with EGF-Rh and observed with a laser-scanning confocal microscope. As shown in Figure 1c, the internalization of EGF-Rh in A431 cells expressing both gp41 and EGFP was significantly higher than that in A431 cells expressing EGFP alone, thus suggesting that gp41 enhances EGF internalization in A431 cells. We then tested whether the HIV-1 envelope glycoprotein (Env) consisting of native

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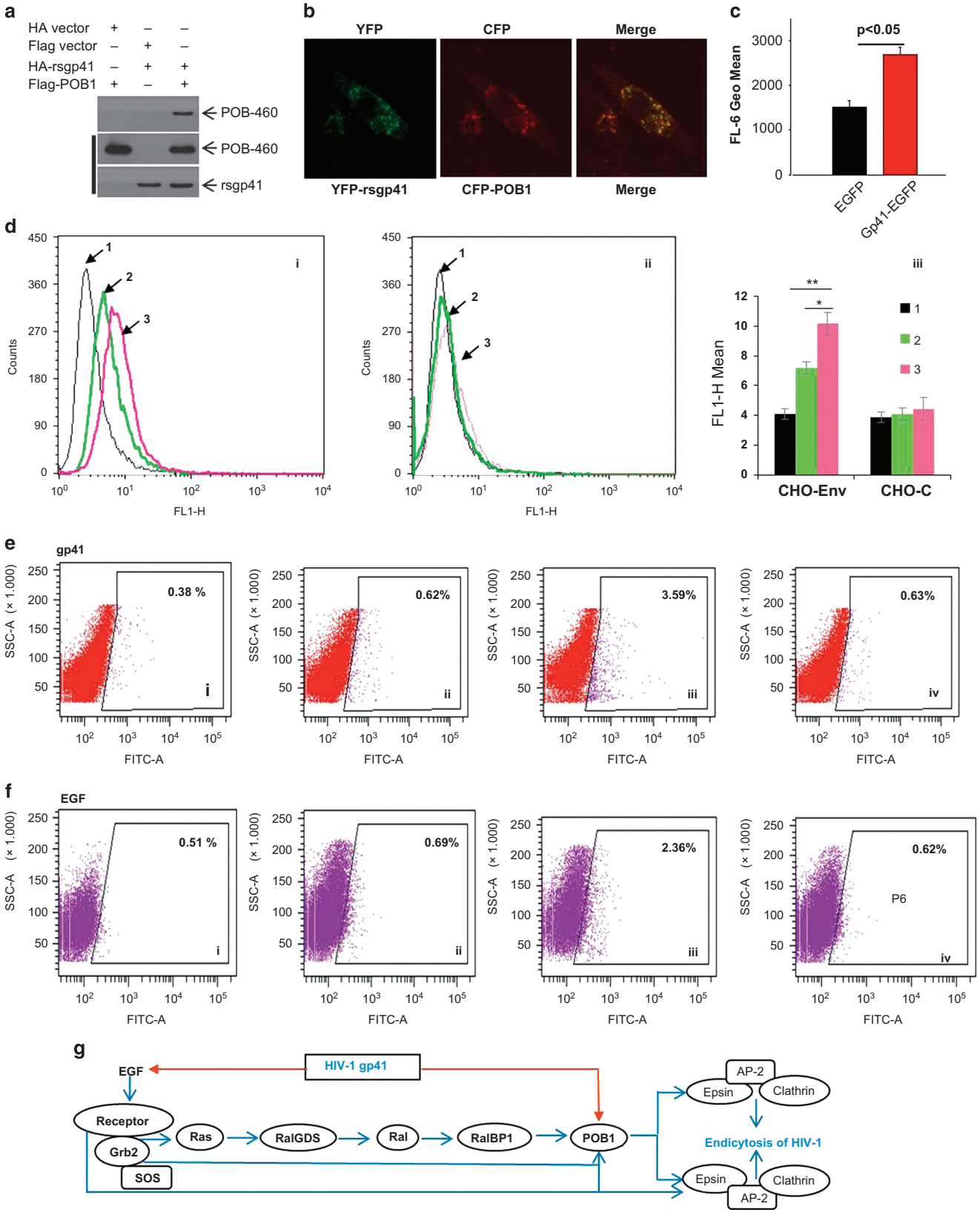
a
HA vector + – –
Flag vector – + –
HA-rsgp41 – + +
Flag-POB1 + – +
POB-460
POB-460
rsgp41
b
YFP
CFP
Merge
YFP-rsgp41
CFP-POB1
Merge
c
p<0.05
FL-6 Geo Mean
3000
2000
1000
0
EGFP
Gp41-EGFP
d
i
ii
iii
Counts
FL1-H
1
2
3
FL1-H Mean
**
*
CHO-Env
CHO-C
e
gp41
SSC-A (× 1.000)
FITC-A
0.38 %
0.62%
3.59%
0.63%
f
EGF
0.51 %
0.69%
2.36%
0.62%
P6
g
EGF
HIV-1 gp41
Receptor
Grb2
SOS
Ras
RalGDS
Ral
RalBP1
POB1
AP-2
Epsin
Clathrin
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gp120 and gp41 subunits can also affect EGF internalization using CHO-Env cells expressing HIV-1 Env upon stimulation with sodium butyrate.[5] Flow cytometric analysis revealed that EGF-Alexa internalization was significantly enhanced in CHO-Env cells after stimulation with sodium butyrate (Figure 1di), whereas no apparent EGF internalization was observed in CHO-C cells lacking HIV-1 Env under the same conditions (Figure 1dii), thus suggesting that the native gp41 in Env can also enhance EGF internalization.

We then studied the effect of gp41 on internalization of EGF and endocytic uptake of HIV-1 virions in A431 cells that were transfected with pECFP-N1-rsgp41-mCherry plasmid. After the addition of the GFP-vpr-labeled HIV-1 virions with or without the endocytosis inhibitor chlorpromazine, cellular fluorescence intensity was measured by flow cytometry. Approximately 0.38 and 0.62% of the cells without gp41 were positive in the absence and presence of HIV-1 virions, respectively (Figure 1ei and eii). Approximately 3.59% of A431 cells expressing gp41 were positive after incubation with HIV-1 virions (Figure 1eiii), whereas only 0.63% of A431 cells were positive after the addition of chlorpromazine, an endocytosis inhibitor (Figure 1eiv). These results suggest that gp41 can promote the entry of HIV-1 virions into CD4-negative epithelial cells, possibly through the endocytosis pathway.

Notably, EGF could also enhance the entry of HIV-1 into CD4-negative A431 cells, as the percentage of positive A431 cells was increased from 0.69% (Figure 1fii) to 2.36% (Figure 1fiii) when EGF was added. Similarly, chlorpromazine could also reverse EGF-mediated enhancement of endocytosis of HIV-1 virions (Figure 1fiv). These results suggest that EGF may non-specifically enhance the endocytosis of HIV-1 virions through POB1.

Extensive evidence has shown that HIV-1 gp41 plays an important role in HIV-1 fusion with CD4-positive immune cells.[6] However, little is known about how gp41 mediates or regulates the endocytic uptake of HIV-1 virions into CD4-negative cells, especially epithelial cells. HIV-1 may enter CD4-negative mucosal epithelial cells before infecting underlying CD4-positive cells through a different mechanism, including endocytosis.[7] Here, we demonstrated that gp41 interacts with POB1 to enhance HIV-1 entry into CD4-negative A431 cells, whereas this enhancement was attenuated by the endocytosis inhibitor, chlorpromazine. Moreover, gp41 could also enhance EGF internalization, which may promote the POB1-mediated endocytosis of HIV-1 (Figure 1f). These findings suggest that HIV-1 gp41 may upregulate POB1-mediated endocytosis by directly binding with POB1 or indirectly interacting with EGF.

The POB1-binding sites in gp41 may serve as targets for future therapeutics or prophylactics against HIV/AIDS. We have previously shown that the NHR-binding peptides derived from the HIV-1 gp41 CHR region, such as SJ-2176,[8,9] are potent HIV-1 fusion inhibitors. The analog peptide T20 [9] was approved by the USFDA as the first HIV fusion inhibitor for use in clinics. We believe that these peptides, by binding with POB1, may be effective in attenuating gp41-mediated enhancement of HIV-1 endocytosis. Similarly, the gp41-binding sites in POB1 may also serve as a target for developing anti-HIV therapeutics or prophylactics. For example, we previously identified a 60-amino-acid fragment (aa462–521) of POB1, designated C60, which bound with the gp41 NHR and the 6-HB formed by NHR and CHR and blocked HIV-1 fusion with the target cell,[1] thus having the potential to be developed as a therapeutic or prophylactic for the treatment or prevention of HIV infection.

Figure 1 (**a**) Co-immunoprecipitation of HIV-1 gp41 and POB1. Lysates of 293T cells co-transfected with pCMV-Flag-POB1–460 and pCMV-HA-rsgp41 or control plasmids were incubated with anti-HA mAb and precipitated with protein A/G beads. The co-immunoprecipitated Flag-POB1–460 was then detected with an anti-Flag mAb. The corresponding bands in the gels indicate that both Flag-POB1–460 and HA-rsgp41 were highly expressed in the co-transfected cells. (**b**) Colocalization of HIV-1 gp41 and POB1 in NRK cells. NRK cells were co-transfected with pEYFP-N1-rsgp41 and pECFP-N1-POB1 and observed with a laser scanning confocal microscope. (**c**) FACS analysis of internalized EGF-Rh in the transfected cells. The average red fluorescence (EGF-Rh) intensity in 1×10^4 of A431 cells expressing gp41-EGFP was $2.69\pm0.88\times10^3$, whereas for control cells expressing EGFP it was $1.51\pm0.15\times10^3$. (**d**) FACS analysis of internalized EGF-Alexa in the CHO-Env and CHO-C cells. EGF-Alexa internalization in CHO-Env cells expressing HIV-1 Env (i) and CHO-C cells expressing no HIV-1 Env (ii) was semi-quantitated by measuring fluorescent density (iii) Statistic analysis for Flow cytometric data. FACS analysis of internalized EGF-Alexa in the CHO-Env and CHO-C cells has been repeated for three times. $*,**p<0.05$." Curve 1, cells without treatment with EGF-Alexa; Curve 2, cells treated with EGF-Alexa; Curve 3, cells pre-stimulated with 6.5 mM sodium butyrate for 20 h and then treated with EGF-Alexa. (**e**) Effect of HIV-1 gp41 on the endocytic uptake of HIV-1 virions in CD4-negative A431 cells. (i) A431 cells were transfected with pECFP-N1-mCherry plasmids as a negative control; (ii) A431 cells transfected with pECFP-N1-mCherry plasmids were incubated with GFP-vpr-labeled HIV-1 virions as a positive control; (iii) A431 cells transfected with pECFP-N1-rsgp41-mCherry plasmids were incubated with GFP-vpr-labeled HIV-1 virions; (iv) A431 cells transfected with pECFP-N1-rsgp41-mCherry plasmids were incubated with HIV-1 virions and chlorpromazine. (**f**) The effect of EGF on the endocytic uptake of HIV-1 virions by CD4-negative A431 cells. (i) A431 cells as a negative control; (ii) A431 cells were incubated with GFP-vpr-labeled HIV-1 virions as a positive control; (iii) A431 cells incubated with 300 ng/ml EGF were incubated with GFP-vpr-labeled HIV-1 virions; (iv) A431 cells incubated with 300 ng/ml EGF were incubated with HIV-1 virions and chlorpromazine. (**g**) The putative mechanism by which gp41 enhances the endocytic uptake of HIV-1 virions in CD4-negative epithelial cells. Binding of EGF with its receptor causes a cascade of signal transduction events, resulting in the enhancement of POB1-mediated clathrin-dependent endocytosis. HIV-1 gp41 may enhance endocytosis of HIV-1 by directly binding with POB1 or indirectly affecting POB1 through internalized EGF.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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