

Short Communication

Correspondence
Ronald C. Desrosiers
ronald_desrosiers@
hms.harvard.edu

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PRA1 co-localizes with envelope but does not influence primate lentivirus production, infectivity or envelope incorporation

Philippe Blancou,† David T. Evans and Ronald C. Desrosiers

New England Regional Primate Research Center, Harvard Medical School, One Pine Hill Drive, Southborough, MA 01772-9102, USA

The results of yeast and mammalian two-hybrid assays previously indicated complex formation between prenylated Rab acceptor 1 (PRA1) and the cytoplasmic domain of gp41 (gp41CD) for both the human and simian immunodeficiency viruses [Evans, D. T., Tilman, K. C. & Desrosiers, R. C. (2002). *J Virol* **76**, 327–337]. The assembly and release of infectious virus particles was studied under conditions of PRA1 overexpression in a transient transfection assay or suppression by RNA interference. Although a clear pattern of co-localization of PRA1 and gp41 was observed, no changes in virion release, infectivity or envelope content were observed as a result of either PRA1 suppression or overexpression. These data show that PRA1 co-localizes with gp41 inside cells and they are consistent with a direct or indirect interaction between these proteins. However, variation in the levels of PRA1 expression did not influence virion production, infectivity or envelope incorporation under the conditions of these assays.

Incorporation of envelope (Env) is essential for the formation of infectious viral particles. Signal sequences in the cytoplasmic domain (CD) are responsible for the high rate of endocytosis of Env protein from the cell surface (Berlioz-Torrent *et al.*, 1999; Boge *et al.*, 1998). The CDs of Env glycoprotein gp41 of human and simian immunodeficiency viruses (HIV and SIV) were previously shown to interact with cellular proteins that participate in vesicular trafficking, including AP-2-clathrin adaptor complexes (Berlioz-Torrent *et al.*, 1999; Boge *et al.*, 1998) and AP-1 complexes (Wyss *et al.*, 2001). The CDs of SIV and HIV-1 contain multiple endocytosis signals of the type YXXΦ and LL that bind to clathrin adaptors and target proteins for endocytosis (Berlioz-Torrent *et al.*, 1999; LaBranche *et al.*, 1995; Sauter *et al.*, 1996; Wyss *et al.*, 2001). More recently, interactions between widely divergent lentiviral Env CDs with prenylated Rab acceptor 1 (PRA1) were identified using mammalian two-hybrid assays (Evans *et al.*, 2002), suggesting a possible role for PRA1 in lentivirus replication.

Rab effector plays a regulatory role in vesicular fusion processes, notably in exocytic and endocytic vesicle transport pathways (Gournier *et al.*, 1998; Pind *et al.*, 1994; Turner *et al.*, 1997). PRA1 has been identified as a Rab partner (Martincic *et al.*, 1997) that inhibits the removal of Rab from the membrane (Hutt *et al.*, 2000), suggesting that

recycling of Rab depends on the action of PRA1, with PRA1 favouring membrane retention of Rab active form. Mutations in the PRA1 gene can result in dramatic disruption of Golgi morphology and cellular trafficking (Gougeon *et al.*, 2002). PRA1 has also been shown to interact with a variety of viral proteins including the cauliflower mosaic virus (CaMV) movement protein (Huang *et al.*, 2001), the Epstein–Barr virus Bcl-2 homologue (Li *et al.*, 2001), the rotavirus V4 spike protein (Enouf *et al.*, 2003) and the SIV envelope protein gp41 (Evans *et al.*, 2002). The selective interaction of PRA1 with Bcl-2 homologue has been shown to reduce its anti-apoptotic activity. However, the functional significance *in vivo* of the interaction between PRA1 and viral proteins remains unexplored for CaMV, rotavirus and primate lentiviruses.

To determine whether PRA1 has an effect on the assembly and release of infectious virus particles, we produced SIV by transient transfection under conditions of PRA1 suppression or overexpression. Although clear co-localization of PRA1 and gp41 could be observed, no change in virion release, infectivity or envelope content was detected.

In order to investigate whether the interaction between PRA1 and primate lentivirus gp41CD observed previously in two-hybrid assays has an effect on virion release, we examined viral production in the context of PRA1 overexpression. 293T cells were transfected with full-length SIVmac239ΔnefEGFP or HIV-1 NL4-3 together with PRA1/Myc-expressing vector or with empty vector

†Present address: Unité d'immunologie-endocrinologie cellulaire et moléculaire, Ecole nationale vétérinaire de Nantes, BP 40706, 44307 Nantes Cedex 3, France.

(pEF1/Myc-HisA; Invitrogen). HIV-1 NL4-3 full-length plasmid was obtained from the NIH AIDS Research Reagent Program and SIVmac239 Δ nefEGFP full-length plasmid is an engineered recombinant derivative of SIVmac239 that expresses enhanced green fluorescent protein (EGFP) (Alexander *et al.*, 1999). All transfections were performed using 1 ml calcium phosphate from the PROFECTION mammalian transfection system (Promega) combined with either 5 μ g viral plasmid or with the PRA1/Myc plasmid or empty vector (10 μ g) added on 293T cell monolayers plated the previous day at 10^6 cells per 100 mm dish. Two days post-transfection, cells and supernatant were harvested to check for PRA1 expression and virion release. The transfection efficiency ranged from 50 to 70% GFP-positive cells and was not affected by transfection with PRA1/Myc (data not shown). PRA1 expression was induced from undetectable levels to a detectable band on a Western blot (Fig. 1a) without apparent cytotoxic effects, as assessed by Trypan blue. Virus release was measured by quantifying the release of capsid protein (p27^{gag} for SIV or p24^{gag} for HIV) in the supernatant 2 days post-transfection using an antigen capture assay (from Coulter for p27^{gag} and from Zepetmetrix for p24^{gag}). Virus production from empty-vector-transfected 293T cells and PRA1/Myc-transfected cells did not show any significant difference (Fig. 1a).

Since PRA1 is involved in the regulation of Golgi trafficking, it is expected that the overexpression of PRA1 will affect envelope incorporation. This could lead to normal levels of virus production but impaired viral infectivity, since virion infectivity may depend on how much envelope protein is incorporated into the viral particle (Yuste *et al.*, 2004). We therefore used various amounts of supernatant from SIVmac239 Δ nefEGFP transfections to infect an immortalized rhesus macaque T cell line (Rh221-89 cells). The infectivity titre was determined 4 days post-infection by the percentage of GFP-positive cells as a function of increasing viral input. No differences in infectivity of the supernatant were noted when SIVmac239 Δ nefEGFP was co-transfected with empty vector [0.795 ± 0.283 % GFP-positive Rh221-89 cells (ng p27)⁻¹] compared with co-transfection with the PRA1-expressing plasmid [0.787 ± 0.144 % GFP-positive cells (ng p27)⁻¹] (Fig. 1b). HIV-1 NL4-3 infectivity was measured by infecting T2-SEAP cells harbouring a Tat-inducible, secreted alkaline phosphatase (SEAP) reporter construct (Means *et al.*, 1997). The amount of SEAP produced in the culture supernatant correlates

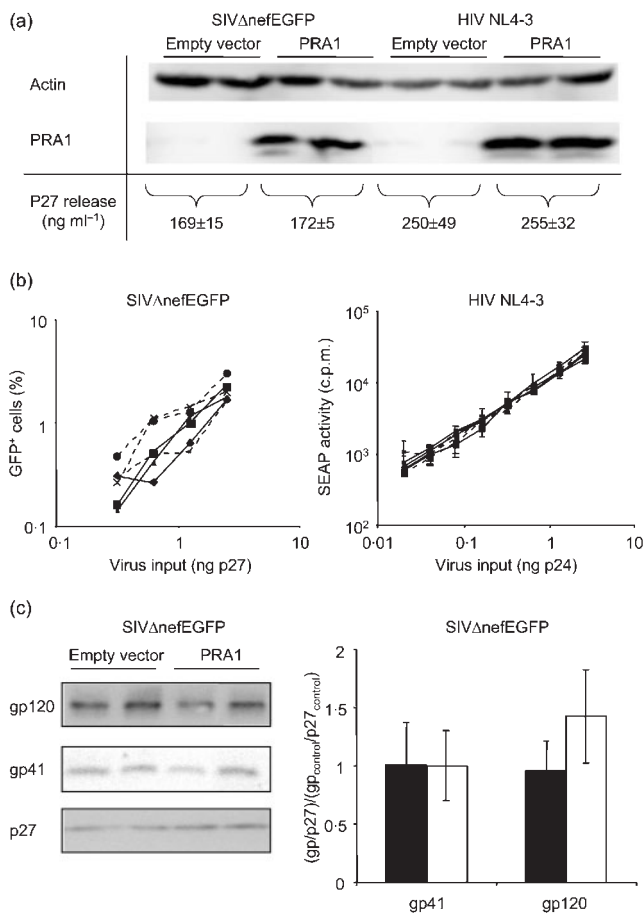


Fig. 1. Effect of PRA1 overexpression on virion release, infectivity and envelope content. 293T cells were co-transfected with PRA1/Myc or empty vector and either with SIVmac239 Δ nefEGFP or full-length HIV-1 NL4-3 plasmids. (a–b) Two days after transfection, cell lysates were checked for PRA1 expression and supernatants were assessed for virion release by p27 or p24 ELISA (a). Infectivity was determined after viral stocks were normalized for p27 or p24 content, showing results for empty vector (solid lines) and PRA1 (dashed lines) (b). (c) Supernatant of SIVmac239 Δ nefEGFP-infected cells was used to infect Rh221-89 cells. SIV infectivity was determined by quantification of GFP-positive cells 4 days post-infection. HIV supernatants were used for triplicate infections of T2-SEAP indicator cells, which contain a Tat-inducible SEAP reporter construct. SEAP activity was measured 4 days post-infection with a Phosphalight kit (Applied Biosystems) according to the manufacturer’s recommendations. All transfections were done in triplicate. Each transfection was then processed separately to determine infectivity. T2-SEAP cells were infected in triplicate. Error bars represent the standard deviation of these triplicates. For envelope content determination, virus was produced by transfection into 293T cells and virions were pelleted from clarified supernatant. p27 contents were determined by ELISA and identical quantities of p27 were loaded for each lane. gp120, gp41 and p27 were detected by Western blotting using mAbs 3.11H, KK41 and 2F12 to probe the same membrane (left panel). Signals were quantified with a 252 Fujimac light scanner. Amounts of gp41 and gp120 were normalized to p27 (referred to as gp/p27) and results are expressed as the relative ratio of envelope content in virions normalized to glycoprotein ratios obtained with supernatant resulting from empty vector co-transfection [referred to as (gp/p27)/(gp_{control}/p27_{control})] (right panel). The results of two independent transfections with empty vector (filled bars) and PRA1 (open bars) are presented. Error bars represent the range of values for these duplicates.

directly with the amount of infecting virus and can be measured sensitively and directly by chemiluminescence assay (Means *et al.*, 1997). Four days post-infection, SEAP activity was determined in the culture supernatant. The infectivity ranged from 9188 ± 1527 c.p.s. $(\text{ng p24})^{-1}$ for supernatant originating from empty-vector-transfected 293T cells to 10054 ± 929 c.p.s. $(\text{ng p24})^{-1}$ for supernatant originating from PRA1/Myc-transfected 293T cells (Fig. 1b).

Thus, PRA1 overexpression did not appear to influence viral infectivity. However, subtle changes in envelope content are sometimes not reflected by infectivity (Yuste *et al.*, 2004).

To examine further the effect of PRA1 overexpression on envelope protein distribution at the surface of the virion, SIVmac239 Δ nefEGFP was concentrated by ultracentrifugation from the supernatant of 293T cells co-transfected either with empty vector or with PRA1/Myc expression plasmid following the protocol described by Yuste *et al.* (2004). The envelope content of the virus produced under different conditions described above was assessed by Western blot from two independent transfections as described by Yuste *et al.* (2004). Reactive mAbs to the ectodomain of transmembrane protein (clone KK41; NIH AIDS Research and Reference Reagent Program), to the V3 loop of gp120 (clone 3.11H; gift of J. E. Robinson, Tulane University Medical School) and to p27 (clone 2F12; NIH AIDS Research and Reference Reagent Program) were used for detection. Ratios of gp120 to p27 and of gp41 to p27 were calculated by normalizing the amount of each envelope glycoprotein to the total amount of p27 capsid protein (gp/p27). These results are presented as relative ratios of gp/p27 normalized to the ratio obtained from control supernatant produced by co-transfection of SIV/HIV full-length genome with the empty vector (Fig. 1c). No difference in gp41 content in virus particles was observed when virus was produced in the context of PRA1 overexpression compared with endogenous PRA1 expression. A slightly higher proportion of gp120 envelope protein was incorporated into virus particles produced in 293T cells transfected with PRA1/Myc expression plasmid than with the empty vector. However, the differences observed were not significant.

Next, we addressed the question of whether SIV gp41 subcellular localization was redistributed by overexpression of PRA1 protein. To be able to detect PRA1 protein in our assays, we developed an anti-PRA1 polyclonal antibody by immunizing rabbits with a purified GST-PRA1 protein. Western blot analysis showed that serum from the immunized rabbit was able to recognize the same band as that observed with anti-myc antibody from lysates of 293T cells transfected with a myc-tagged PRA1 expression construct (data not shown). This serum was also able to recognize cellular protein by immunostaining when cells were transfected with a PRA1-GFP construct (data not shown). The perinuclear punctate subcellular distribution observed for PRA1 is consistent with the association of wild-type PRA1 protein with the Golgi complex as described previously

(Gougeon *et al.*, 2002). However, all attempts to detect endogenous PRA1 in untransfected cells by Western blot or immunohistostaining failed, even when cellular proteins were immunoprecipitated with anti-PRA1 serum (data not shown).

To ascertain the PRA1 localization relative to gp41 distribution, 293T cells were co-transfected with SIVmac239 Δ nefEGFP and PRA1/Myc expression plasmid and stained 2 days later with 1:250 mouse anti-gp41 antibody (KK41) and 1:50 anti-PRA1 rabbit polyclonal serum at the same time. The results analysed by confocal microscopy are shown in Fig. 2. Transfected cells were identified by GFP expression from the *nef* locus. The PRA1 protein was visualized with the specific rabbit antiserum coupled to goat anti-rabbit Alexa 568 (Molecular Probes), appearing red by confocal microscopy, whereas gp41 was visualized with goat anti-mouse 633 antibody (Molecular Probes), appearing blue. gp41 showed essentially the same perinuclear punctate distribution that has been described with HIV-1 (Boge *et al.*, 1998; Wyss *et al.*, 2001). Clear co-localization of PRA1 and gp41 can be observed in 293T cells, suggesting an interaction between these two proteins (Fig. 2). However, the subcellular distribution of gp41 showed no consistent differences in PRA1/Myc-overexpressing cells compared with cells transfected with the empty vector (Fig. 2).

The results of PRA1 overexpression experiments indicate that, even though PRA1 co-localized with gp41, it did not seem to affect gp41 subcellular localization, viral shedding at the cell surface or infectivity of viral particles.

Since endogenous PRA1 may be sufficient for any role that PRA1 might play in virus replication, PRA1 overexpression may not alter virus production or envelope incorporation. To ascertain whether the loss of PRA1 expression has an effect on SIV or HIV replication, we investigated virus production under conditions of PRA1 suppression by using RNA interference.

The protocol of PRA1 suppression was adapted from Elbashir *et al.* (2001). PRA1 interfering RNA (RNAi) spanning positions 384–404 on the PRA1 mRNA (GenBank accession no. NM_006423) was synthesized by Dharmacon (Lafayette, CO, USA). For control experiments, an RNAi containing a two-nucleotide mismatch (in bold) with respect to the PRA1 sequence was used as target (5'-GCT-TGTGCTCCCTGGCCGAGA). Suppression of PRA1 was confirmed by co-transfecting the RNAi oligonucleotides with the PRA1-GFP expression construct. PRA1 expression was reduced by 65–90% relative to controls at 48 and 72 h post-transfection (data not shown).

Virus production was assessed in 293T cells 3 days after co-transfection of full-length SIVmac239 Δ nefEGFP or HIV-1 NL4-3 with control or anti-PRA1 RNAi oligonucleotides. Endogenous PRA1 protein expression could not be detected by Western blot. However, to confirm that

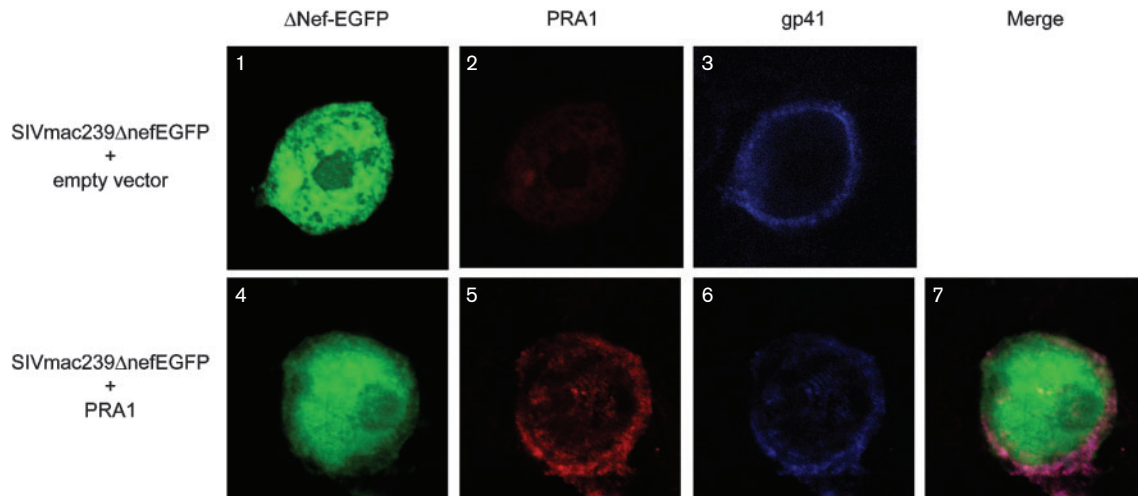
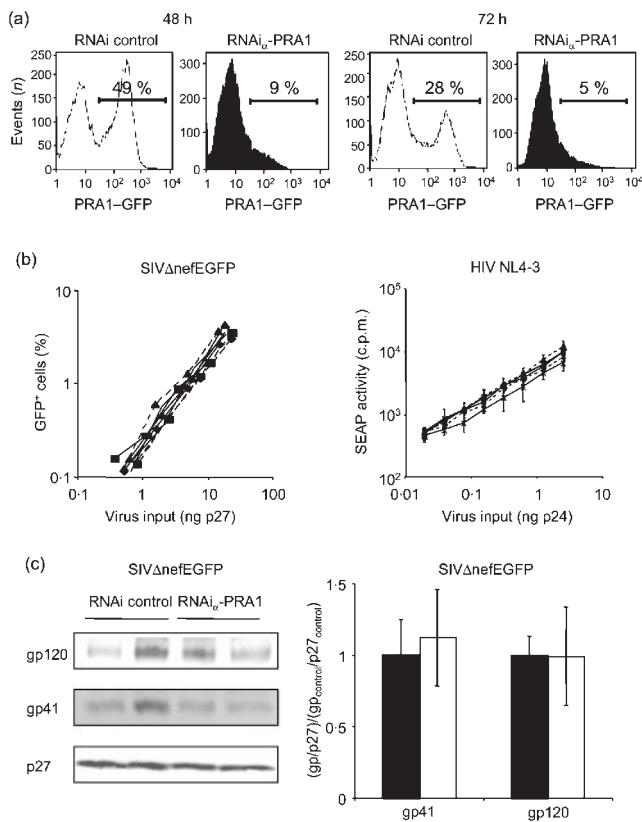


Fig. 2. Effect of PRA1 overexpression on subcellular localization of gp41. 293T cells were co-transfected with full-length SIVmac239ΔnefEGFP plasmid and with either empty vector or PRA1Myc plasmid. Two days post-transfection, the cells were permeabilized and stained with anti-PRA1 rabbit serum and KK41 anti-gp41 antibody and then revealed by Alexa 568 Polyclonal anti-rabbit Ig (red) and Alexa 633 anti-mouse Ig G (blue). Cells were then analysed by confocal microscopy. Magnification, $\times 100$.

PRA1 expression was suppressed, GFP expression in PRA1-GFP/RNAi-co-transfected 293T cells was followed in parallel under the same conditions. In this assay, a decrease of more than 80 % was observed in 293T cells co-transfected with PRA1-GFP plasmid and anti-PRA1 RNAi



compared with control transfection (Fig. 3a). Under these conditions, we assessed virus production of SIVmac239gfp and HIV-1 NL4-3 by antigen-capture ELISA. Detection of p27 protein in the culture was similar for anti-PRA1 and control oligonucleotides (96 ± 16 and 105 ± 10 ng p27 ml⁻¹, respectively). The same was true for HIV-1 NL4-3-transfected 293T cells, for which p24 production in the culture supernatant was independent of the presence of anti-PRA1 RNAi (162 ± 8 ng p24 in the presence of control RNAi compared with 167 ± 16 in the presence of anti-PRA1 RNAi).

As in the previous experiment, the infectivity of supernatant from SIVmac239ΔnefEGFP-transfected 293T cells was assessed by infecting Rh221-89 cells and by quantifying GFP-positive cells. The percentage of GFP-positive Rh221-89 cells was similar when these cells were infected with supernatant originating from control RNAi-co-transfected cell compared with supernatant originating from anti-PRA1 RNAi-co-transfected cells [0.194 ± 0.044 %

Fig. 3. Effect of PRA1 suppression on virion release, infectivity and envelope content. 293T cells were co-transfected with either anti-PRA1 RNAi primers or control RNAi and either with full-length SIVmac239ΔnefEGFP or HIVNL4-3 plasmids. (a–b) Two days after transfection, the effect of RNAi was assessed by FACS analysis (a). Infectivity was determined after viral stocks were normalized for p27 or p24 content, with either control RNAi (solid lines) or anti-PRA1 RNAi primers (dashed lines) (b). SIV infectivity, SEAP activity and envelope content were determined as outlined in Fig. 1; Western blot results (left panel) and normalized results (right panel: RNAi control, solid bars; anti-PRA1 RNAi, open bars) are shown (c).

compared with 0.186 ± 0.058 % GFP-positive cells (ng p27)⁻¹; Fig. 3b]. The infectivity of HIV-1 NL4-3 virus aliquots from 293T cells infected with control RNAi or with anti-PRA1 RNAi showed the same trend [3805 ± 786 compared with 4422 ± 1020 c.p.s. (ng p24)⁻¹; Fig. 3b].

Finally, envelope content of SIVmac239 Δ nefEGFP virions measured as band intensity of gp41 or gp120 from viral particles in a Western blot showed no significant differences when control RNAi or anti-PRA1 RNAi primers were used. The relative ratio of gp41 and gp120 was not statistically different when the virus was produced in the presence of anti-PRA1 RNAi or control RNAi (1 : 1.176 for gp41 and 1 : 0.934 for gp120; Fig. 3c).

The contribution of SIV and HIV gp41CD to virus replication is highly cell-type-dependent (Murakami & Freed, 2000). Passage of SIV in human T-cell lines selects for a premature stop codon that truncates the gp41CD (Kodama *et al.*, 1989), whereas viruses with a truncation rapidly revert to restore the full-length gp41CD during replication in cultured macaque peripheral blood mononuclear cells (PBMC) or in infected animals (Kodama *et al.*, 1989). This suggests that the full-length gp41CD confers a selective advantage on SIV replication in macaque PBMC. Thus, the inability to observe an effect of PRA1 on virus replication may reflect the known dispensability of the SIV gp41CD for replication in certain cell types. Moreover, our results are limited to the cells and conditions of our *in vitro* measurements. PRA1 interaction may be important for envelope trafficking and virion incorporation under conditions of much more limited env expression than was present in our experiments. Although the natural promoter was used to drive gp41 gene expression in our experiments, 293T cells may produce different amounts of protein than the natural SIVmac239 targets. In addition, it is possible that the cellular physiology of gp41CD with respect to different cellular patterns may vary with cell type, state of activation or environment.

The transmembrane envelope glycoprotein of HIV-1 co-localizes in a perinuclear area in association with *trans*-Golgi network markers (Boge *et al.*, 1998; Wyss *et al.*, 2001) as a result of a retrograde transport from the cell surface to the *trans*-Golgi network (Rowell *et al.*, 1995; Sauter *et al.*, 1996). In support of this concept, it has recently been shown that HIV gp41CD interacts with TIP47, a protein required for the transport of mannose-6-phosphate receptors from the endosome to the *trans*-Golgi network (Blot *et al.*, 2003). SIV gp41 also co-localizes with *trans*-Golgi network markers, which could lead to the co-localization of PRA1 and gp41 observed by confocal microscopy without biological effects on the SIV life cycle. It is possible that the PRA1 interaction with gp41 revealed by two-hybrid assay and by confocal co-localization may not reflect a direct interaction, but PRA1 may only be a participant in a multiprotein complex involving gp41.

Finally, the absence of any effect could also be due to a subtle

role of PRA1 on gp41 migration. For example, it has been shown that overexpression of PRA1 has a minimal effect on the transport of vesicular stomatitis virus envelope protein from the cytoplasm to the membrane (Gougeon *et al.*, 2002); a migration delay of only about 30 min was detected when wild-type PRA1 was overexpressed. Since we examined viral release 24 h after transfection, it is possible that this lag is not sufficient to detect any effect of PRA1 on virion release.

Our data suggest that, although PRA1 and SIV gp41CD co-localize, there is no measurable effect on HIV-1 or SIV virion release, infectivity or envelope incorporation under conditions of PRA1 overexpression or suppression in culture cells. Moreover, a very sensitive method based on the detection of the envelope at the surface of the virion failed to show any differences when endogenous levels of PRA1 were altered.

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