Available online at www.sciencedirect.com
SCIENCE DIRECT
Virology 347 (2006) 217 – 225
VIROLOGY
www.elsevier.com/locate/yviro

Mutations at the C-terminus of the simian immunodeficiency virus envelope glycoprotein affect gp120-gp41 stability on virions

José L. Affranchino [1], Silvia A. González *

Centro de Virología Animal (CEVAN-CONICET), Serrano 669, C1414DEM Buenos Aires, Argentina

Received 14 October 2005; returned to author for revision 27 October 2005; accepted 22 November 2005
Available online 27 December 2005

Abstract

The transmembrane (TM) subunit of the envelope (Env) glycoprotein of the simian immunodeficiency virus (SIV) contains an unusually long cytoplasmic domain of 164 amino acids. Previously, we identified domains in the SIV TM cytoplasmic tail that are necessary for Env incorporation into virions and viral infectivity. In this study, we investigated the relevance to Env function of the highly conserved sequence comprising the immediate C-terminal 19 residues of TM. To this end, small in-frame deletions as well as a premature stop codon mutation were introduced into the coding region for the SIV TM C-terminus. All the mutant Env glycoproteins were expressed, processed and transported to the cell surface in an essentially wild-type manner. Moreover, the ability of the mutant Env proteins to mediate cell-to-cell fusion was similar to or slightly lower than that of the wild-type Env. However, viruses expressing the mutant Env glycoproteins were found to be poorly infectious in single-cycle infectivity assays. Further characterization of the TM mutant viruses revealed that while exhibiting wild-type levels of the TM protein, they contained significantly lower levels of the Env surface (SU) subunit, which is consistent with increased SU shedding from virions after Env incorporation. This phenotype was independent of Gag processing, since genetic inactivation of the viral protease did not increase SU retention by the resulting immature particles. Our findings indicate that deletions at the C-terminus of the SIV Env promote the instability of the SU-TM association on the virion surface and point to an important role for the TM cytoplasmic domain in modulating Env structure.

Keywords: Simian immunodeficiency virus; Envelope glycoprotein; gp120; gp41; Cytoplasmic domain; Virus infectivity

Introduction

The envelope (Env) glycoproteins of human and simian immunodeficiency viruses (HIV and SIV, respectively) are synthesized as a precursor that is proteolytically cleaved by cellular proteases during its trafficking to the cell surface (Hunter, 1997). Cleavage of the Env precursor yields the surface (SU, gp120) and the transmembrane (TM, gp41) glycoproteins that remain noncovalently associated in the form of trimers of heterodimeric complexes that are incorporated into budding virus particles. Incorporation of the SU-TM complexes into virions is essential for virus infectivity since the SU glycoprotein binds to the CD4 and chemokine receptors present at the surface of target cells, whereas the TM subunit induces the fusion of the viral and cellular membranes during virus entry (Chan and Kim, 1998).

The TM glycoproteins of HIV and SIV consist of an ectodomain containing a fusion peptide, a membrane anchor and a carboxy-terminal cytoplasmic tail which, in contrast to that of most retroviral TM proteins, is unusually long: about 150 and 164 amino acids for HIV-1 and SIV, respectively.

The SIV TM cytoplasmic tail plays a key role in important viral functions, such as the regulation of Env expression at the cell surface and the incorporation of Env into virions. In this regard, the SIV TM cytoplasmic domain contains multiple motifs that allow clathrin-dependent Env endocytosis (Berlioz-Torrent et al., 1999; Bowers et al., 2000; LaBranche et al., 1995; Sauter et al., 1996). Moreover, we have presented evidence indicating that an interaction between the SIV TM cytoplasmic tail and the matrix (MA) domain of the viral Gag

* Corresponding author. Laboratorio de Virología, Facultad de Ciencias Exactas y Naturales, Universidad de Belgrano (UB), Villanueva 1324, C1426BMJ Buenos Aires, Argentina. Fax: +54 11 4963 9278.
E-mail address: sigonzal@ub.edu.ar (S.A. González).
[1] Present address: Laboratorio de Virología, Facultad de Ciencias Exactas y Naturales, Universidad de Belgrano (UB), Villanueva 1324, C1426BMJ Buenos Aires, Argentina.

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.11.032

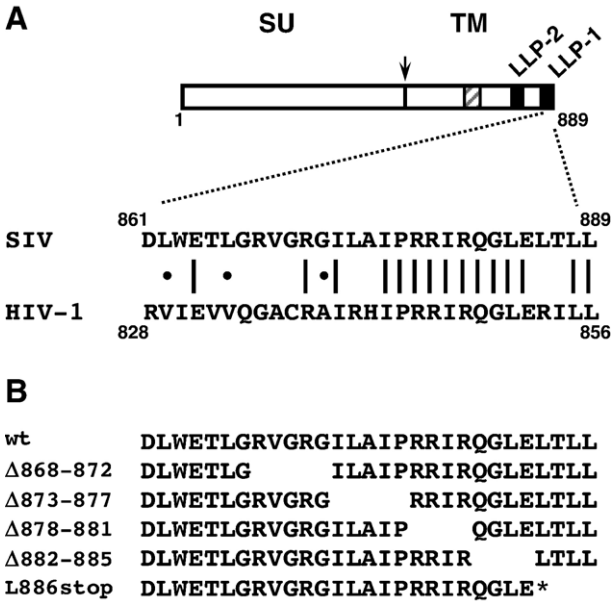

Fig. 1. Mutagenesis of the C-terminal domain of the SIV TM glycoprotein. (A) The SIV Env protein is depicted at the top showing the cleavage site between its SU and TM subunits (arrow) as well as the location of the TM membrane-spanning domain (shaded box) and the predicted amphipathic α-helices LLP-1 and LLP-2 (black boxes). Amino acid residues are numbered according to their positions in the $SIV_{SMM}PBj1.9$ Env protein. A sequence alignment of the LLP-1 region of SIV PBj1.9 TM protein with the homologous region of HIV-1 HXB2 Env protein is shown. Identical and similar amino acids are denoted by lines and dots, respectively. (B) The in-frame deletions as well as the premature stop codon mutation introduced into the SIV TM C-terminal region are shown, with the name of the mutations listed below the wild-type (wt) Env sequence. The blank spaces correspond to the amino acids deleted in each Env protein. The asterisk denotes the Leu886stop mutation.

polyprotein mediates Env packaging into particles. Indeed, mutations at the SIV MA N-terminus confer a differential ability to Gag to associate with the Env glycoprotein by modulating either negatively or positively the levels at which Env is incorporated into virions (Manrique et al., 2003). In addition, we have shown that small in-frame deletions or truncations affecting the C-terminal third of the SIV TM cytoplasmic tail abolish Env incorporation into particles and virus infectivity (Celma et al., 2001; Manrique et al., 2001). However, truncated SIV Env glycoproteins containing cytoplasmic domains of 44 or 24 amino acids are packaged into virions more efficiently than the wild-type Env and enhance virus infectivity with respect to that conferred by the full-length Env protein (Manrique et al., 2001). Interestingly, the defect in Env incorporation and virus replication caused by a mutation at the C-terminal third of the Env cytoplasmic domain is reversed by the appearance of premature stop codons that truncate the TM cytoplasmic tail to 52 or 48 amino acids (Celma et al., 2004). Taken together, these studies indicate that the length of the Env cytoplasmic domain modulates virus infectivity. This notion is also illustrated by the observation that passage of SIV_{mac} in human T cell lines selects for variants with Env cytoplasmic tails of 18 amino acids (Chakrabarti et al., 1989; Hirsch et al., 1989; Kodama et al., 1989), which correlates with the increased ability of these Env proteins to mediate membrane fusion and virus entry (Johnston et al., 1993; Ritter et al., 1993; Spies and Compans, 1994; Zingler and Littman, 1993). However, when these mutant viruses are inoculated into rhesus macaques, rapid reversion to wild-type viruses encoding full-length Env proteins is observed, which underscores the requirement for a full-length TM cytoplasmic tail for SIV replication in vivo (Kodama et al., 1989; Luciw et al., 1998).

The cytoplasmic tails of the Env glycoproteins from diverse HIV-1 and SIV isolates contain a central and a C-terminal region that would be predicted to fold into amphipathic α-helical segments designated as lentivirus lytic peptides 2 and 1 or LLP-2 and LLP-1, respectively (Miller et al., 1991; Venable et al., 1989). Synthetic peptides corresponding to these TM regions have been shown to bind (Koenig et al., 1995; Srinivas et al., 1992) and perturb (Chernomordik et al., 1994; Comardelle et al., 1997; Miller et al., 1993a) membranes as well as to interact with calmodulin (Miller et al., 1993b; Srinivas et al., 1993; Tencza et al., 1997). It has been proposed that binding of the HIV-1 and SIV TM to calmodulin may interfere with calmodulin-dependent signal transduction pathways operating in T lymphocytes, which may be related to viral cytopathogenesis (Miller et al., 1993b).

In the present report, we further addressed the function of the SIV TM cytoplasmic domain by studying the relevance of the C-terminal 19 residues of Env. This region, which forms part of the LLP-1 domain, is an attractive target for mutagenesis analysis since an almost identical sequence is found in HIV-1 Env despite the fact that the cytoplasmic domains of the HIV-1 and SIV TM proteins exhibit little amino acid sequence similarity. Our results show that mutations within the immediate C-terminal region of the SIV Env cytoplasmic tail impair virus infectivity by destabilizing the association between the SU and TM subunits on the virion surface.

Results

Synthesis, processing, and cell surface expression of mutant SIV Env glycoproteins

To extend our previous studies on the role of the SIV TM cytoplasmic domain in the virus life cycle, we performed a site-directed mutagenesis analysis of the SIV Env C-terminal 19 residues. This Env region is also highly conserved among HIV-1 isolates. As shown in Fig. 1, we introduced five mutations within this region: a premature stop codon that removes the C-terminal 4 amino acids of the Env protein (mutation Leu886stop) and four small in-frame deletions (mutations Δ868–872; Δ873–877; Δ878–881; and Δ882–885).

We first analyzed the effect of these mutations on Env expression and processing by transfection of 293T cells with plasmid constructs encoding Tat, Rev, and either the wild-type or each of the mutant Env glycoproteins (see Materials and methods). Cell lysates as well as the clarified cell culture supernatants were analyzed by Western blotting using an anti-SIV SU monoclonal antibody (MAb). As can be seen in

Fig. 2A, all the mutant Env proteins were expressed at levels comparable to those of wild-type Env and were found to be processed to the SU and TM subunits. In addition, we quantitated the levels of Env proteins in both cell lysates and supernatants to determine the Env precursor processing and SU-TM association indexes of the mutant Env proteins (Fig. 2B). Mutants Δ868–872, Δ873–877, and Δ878–881 were processed in an essentially wild-type manner. Likewise, the stability of the SU-TM association was not significantly affected by these mutations. By contrast, mutants Δ882–885 and Leu886stop were processed less efficiently than the wild-type Env (processing indices of 0.58 and 0.45, respectively). However, these Env mutants exhibited reduced shedding of the SU glycoprotein as inferred from their association indices of 1.54 (mutant Δ882–885) and 1.66 (mutant Leu886stop) with respect to that of wild-type Env (Fig. 2B).

We next assessed the level of surface expression of the mutant Env glycoproteins by surface biotinylation of 293T cells expressing these proteins. In the case of the wild-type Env, and in agreement with our previous studies (Celma et al., 2001; Manrique et al., 2001), surface biotinylation resulted in the labeling of both the SU and Env precursor glycoproteins (Fig. 2C). All of the Env mutants showed a pattern of surface expression similar to that of wild-type Env, indicating that they are efficiently transported to the cell surface (Fig. 2C).

Cell–cell fusion mediated by the mutant Env glycoproteins

SIV entry into its target cells requires the interaction of the Env glycoprotein with the cellular receptor–coreceptor complex followed by the Env-mediated fusion of the viral and cellular membranes. Therefore, Env function can be assessed

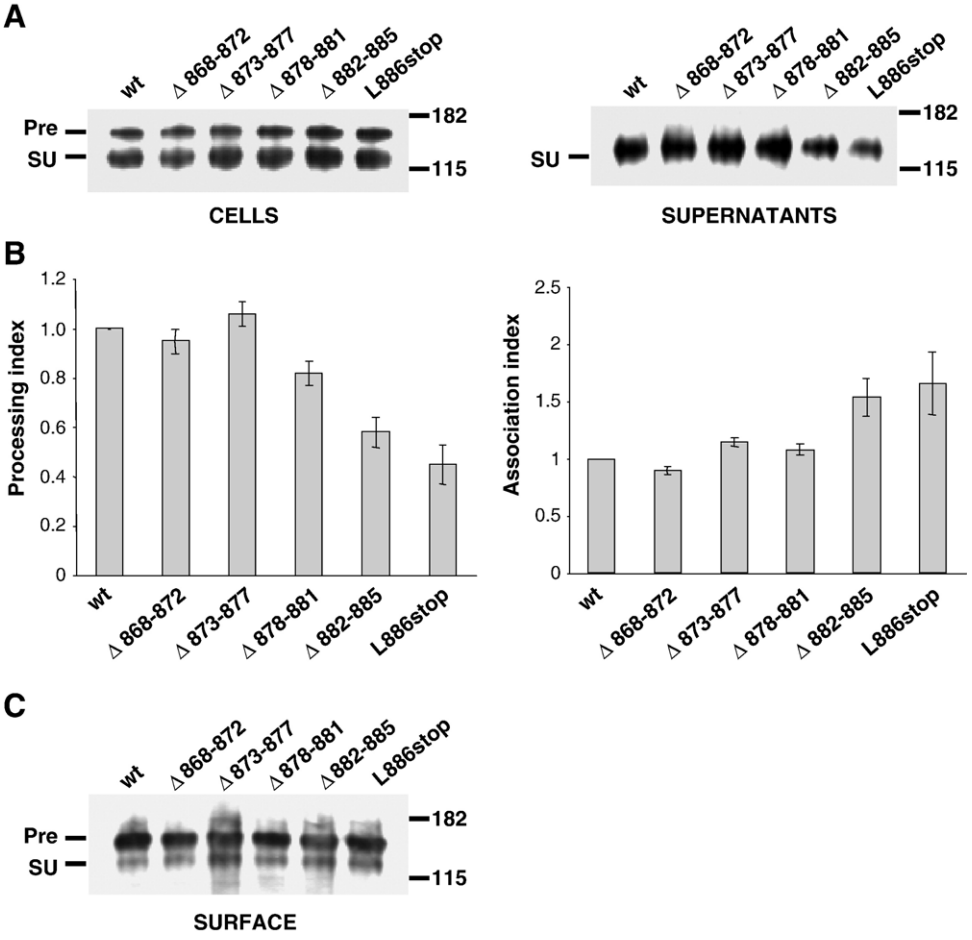

Fig. 2. Biosynthesis, processing, and cell surface expression of mutant SIV Env proteins. (A) The expression plasmids encoding the wild-type (wt) and the mutant Env glycoproteins were transfected into 293T cells. 48 h posttransfection, the Env proteins present in cell lysates (CELLS) and culture supernatants (SUPERNATANTS) were detected by Western blotting using an anti-SIV SU MAb. The mobilities of the viral Env precursor (Pre) and the SU subunit (SU) are shown, as are those of the molecular weight standards. (B) The amounts of Env precursor and SU proteins for wild-type and Env mutants in the cell lysates and culture supernatants were quantitated as described in Materials and methods, and the resulting values were used to calculate the processing and association indices. Processing index: $([\text{total SU}]_{\text{mutant}} \times [\text{Env precursor}]_{\text{wild-type}})/([\text{Env precursor}]_{\text{mutant}} \times [\text{total SU}]_{\text{wild-type}})$, where total SU corresponds to the amounts of SU detected in both cell lysates and supernatants. Association index: $([\text{intracellular SU}]_{\text{mutant}} \times [\text{total SU}]_{\text{wild-type}})/([\text{total SU}]_{\text{mutant}} \times [\text{intracellular SU}]_{\text{wild-type}})$, where intracellular SU denotes the SU levels detected in cell lysates. Data represent mean values and standard deviations of three independent experiments. (C) Cells expressing the wild-type or the mutant Env proteins were biotinylated as described in Materials and methods. Env proteins were immunoprecipitated from cell lysates, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Proteins on the cell surface (SURFACE) were visualized by using streptavidin-conjugated horseradish peroxidase coupled with enhanced chemiluminescence.

by evaluating its ability to mediate cell–cell fusion. To this end, 293T cells were transfected with the *env* expression plasmids and 48 h posttransfection cells were mixed with MAGI-CCR5 cells as described under Materials and methods. Since the *env* expression plasmids also direct the synthesis of the viral Tat protein, fusion of the two cell types induces the expression of the β-galactosidase reporter gene that is under the control of the viral long terminal repeat, thus facilitating syncytia detection. Under these experimental conditions, cells expressing wild-type Env yielded 460 ± 29 blue syncytia per well (average of three assays ± SD). As shown in Table 1, the fusogenic capacity of most of the SIV Env mutants was similar to or slightly higher than that of wild-type Env. The sole exception was the mutant Leu886stop that exhibited an ability to mediate cell–cell fusion representing 75% of the wild-type value.

Infectivity of viruses expressing the mutant Env glycoproteins

To investigate whether the deletions introduced at the C-terminus of the SIV TM cytoplasmic tail affect virus infectivity, the mutated *env* genes were expressed in the context of the infectious molecular clone PBj1.9. Wild-type and mutant proviral DNA constructs were transfected into 293T cells and 48 h posttransfection, the cell culture supernatants were normalized for reverse transcriptase (RT) activity and used to infect MAGI-CCR5 cells. As shown in Fig. 3, mutations Δ868–872, Δ873–877, Δ878–881, and Leu886stop caused a drastic loss of virus infectivity when compared to that of the wild-type virus. Indeed, the relative infectivities of these mutant viruses were reduced by 78%, 93%, 87%, and 82%, respectively. By contrast, the infectivity of mutant Δ882–885 virus was moderately affected, exhibiting an infectivity of 46% of the wild-type value (Fig. 3).

Incorporation of mutant Env glycoproteins into virions

Our results to this point indicated that the mutant Env glycoproteins were highly inefficient at mediating virus infectivity in MAGI assays despite exhibiting a similar phenotype to that of wild-type Env with respect to cell surface expression and fusogenicity. We therefore decided to investigate the cause of the defect in infectivity exhibited by the mutant viruses by analyzing the ability of the mutant Env glycoproteins to be incorporated into virions. 293T cells were transfected with either the wild-type or the mutant proviral constructs, and both the cell and virion lysates were assayed for the presence of viral proteins by Western blotting. The degree of Env incorporation into virions was first evaluated by assessing the levels of virion-associated TM protein. Fig. 4 shows that all the Env mutant viruses packaged the TM protein in an essentially wild-type manner, indicating that the process of Env incorporation into virions was not affected by the mutations introduced at the C-terminus of the SIV TM cytoplasmic tail. However, when the virion samples were probed with an anti-SU MAb, we found that the Env mutants exhibited significantly lower levels of SU than those of wild-type virions (Fig. 4B). Quantitation of wild-type and mutant virion proteins in three independent experiments revealed that the SU levels relative to CA were reduced to 27.5 ± 0.9% (mutant Δ868–872); 9.9 ± 1.8% (mutant Δ873–877); 20.5 ± 2.4% (mutant Δ878–881); 44.8 ± 8.1% (mutant Δ882–885); and 13.0 ± 2.7% (mutant Leu886stop) with respect to those on wild-type virions. Interestingly, a good correlation was observed between the SU content of mutant virions and their MAGI titers (Pearson correlation coefficient of 0.99). Taken together, our results indicate that the deletions introduced at the SIV Env C-terminus promote the instability of the SU-TM association thereby favoring SU shedding from virions. This in turn leads to an impairment in virus infectivity.

Of note, mutation Leu886stop had an additional effect on the Env composition of virions. Indeed, the mutant Env precursor was incorporated into virions at levels significantly

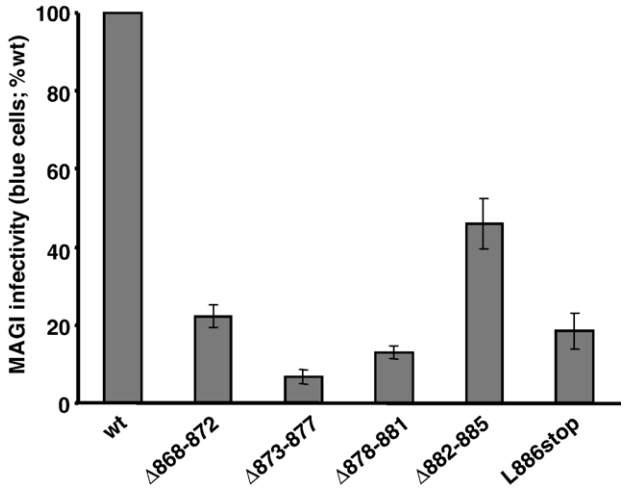

Fig. 3. Effect of mutations at the SIV TM C-terminus on virus infectivity. Virus stocks, obtained by transfection of 293T cells with the wild-type PBj1.9 or the Env mutant proviral clones, were normalized for RT activity and used to infect the MAGI-CCR5 cell line. 48 h postinfection, cells were stained for β-galactosidase activity. Virus entry was quantitated as the total number of blue foci obtained by infection with each mutant virus and referred to that obtained on infection with wild-type PBj1.9 (considered as 100%). Data represent the mean values and standard deviations of three independent experiments.

Table 1
Cell-to-cell fusion mediated by the SIV Env mutant glycoproteins[a]

Env glycoprotein	Relative fusion (%wild-type)[b]
Wild-type	100
Δ868–872	104.0 ± 9.7
Δ873–877	116.8 ± 10.9
Δ878–881	118.6 ± 13.4
Δ882–885	119.7 ± 7.6
Leu886stop	75.8 ± 9.8

[a] 293T cells expressing the wild-type or the mutant Env glycoproteins were dissociated, and equivalent numbers of cells were added at a 1:10 ratio to 4×10^4 MAGI-CCR5 cells. Coculture was continued for 24 h, after which cells were stained for β-galactosidase and scored for syncytia formation.

[b] Data presented were obtained from three independent experiments, and each assay was performed in triplicate. Blue foci were counted in at least 20 fields, and the mean number of syncytia per field was referred to that obtained for wild-type Env; ±standard deviations.

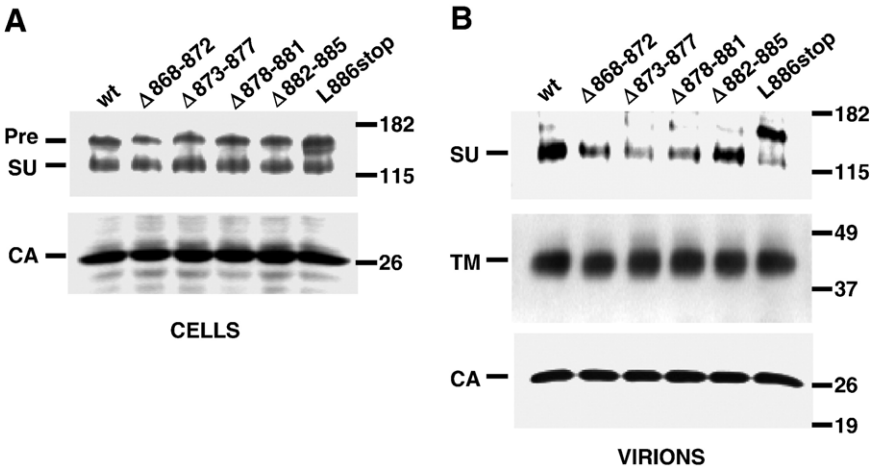

Fig. 4. Effect of mutations at the SIV TM C-terminus on Env glycoprotein incorporation into virions. 293T cells were transfected with wild-type (wt) PBj1.9 or the TM mutant proviral clones. At 48 h posttransfection, viral proteins from cell lysates (A) and virions (B) were analyzed by Western blotting using MAbs specific for the SU, TM, or CA proteins. The mobilities of the viral Env precursor (Pre), SU, TM, Gag, and CA proteins are shown, as are those of the molecular weight standards.

higher than those of the wild-type Env precursor (Fig. 4B). Since the levels of unprocessed Leu886stop Env on the cell surface are similar to those of the wild-type Env (Fig. 2C), it may be speculated that this mutation allows the Env precursor to bypass the exclusion mechanism by which budding virions selectively incorporate the fully processed Env protein complex (Dubay et al., 1995).

Incorporation of the mutant Δ873–877 Env into immature SIV particles

It has been reported that coexpression of HIV-1 Env with an immature Gag core results in enhanced retention of the SU glycoprotein in pseudovirions (Hammonds et al., 2003). We therefore decided to investigate whether the instability of the

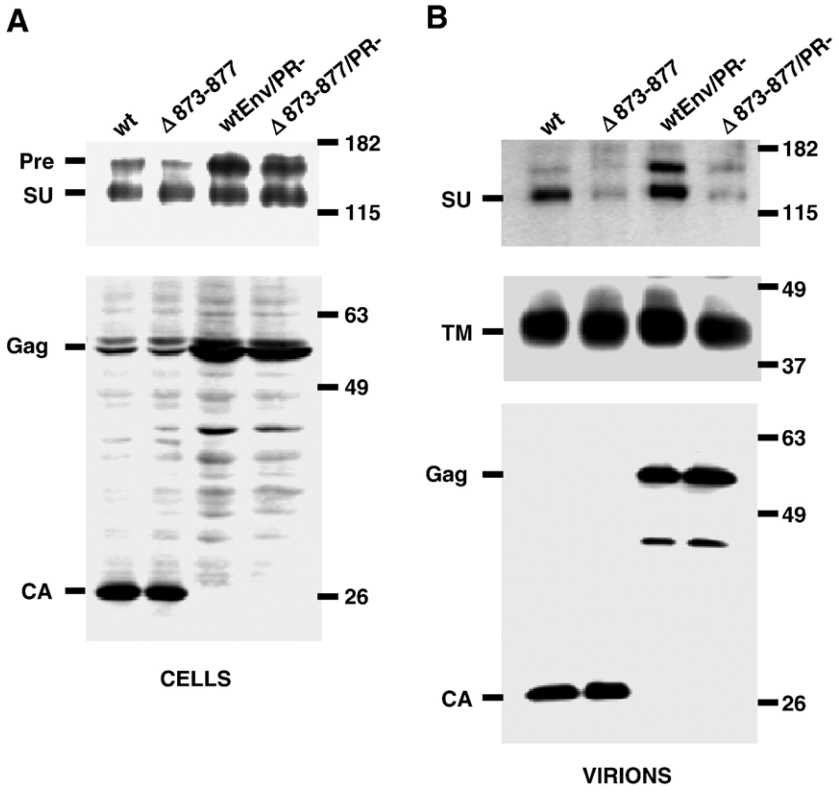

Fig. 5. Incorporation of the SIV Δ873–877 Env mutant glycoprotein into immature virions. 293T cells were transfected with the wild-type Env-PR$^-$ and Δ873–877 Env-PR$^-$ proviral DNAs. At 48 h posttransfection, cells were harvested, and virus particles were purified from the clarified culture supernatants. Viral proteins from cell lysates (A) and virions (B) were detected by Western blotting using MAbs specific for the SU, TM, or CA proteins. The mobilities of the viral Env precursor (Pre), SU, TM, Gag, and CA proteins are shown.

SIV SU protein on the virion surface caused by the deletions introduced into the Env C-terminus could be reversed by incorporation of the Env mutants into unprocessed Gag particles. To this end, we generated SIV proviruses containing either the wild-type or the mutant Δ873–877 *env* genes together with inactivating amino acid substitutions at the viral protease (PR) active site: the wild-type Env-PR$^-$ and the Δ873–877 Env-PR$^-$ mutants, respectively. We chose the Δ873–877 mutant because it exhibited the most drastic defect in both SU stability and virus infectivity. 293T cells were transfected with the wild-type Env-PR$^-$ and Δ873–877 Env-PR$^-$ proviral DNAs in parallel with the wild-type and mutant Δ873–877 Env parental clones. Cell and virion lysates were analyzed for viral proteins by Western blotting. As shown in Fig. 5A, transfection of the PR$^-$ clones resulted in the expression of the Gag precursor without any detectable CA protein, which confirmed the inactivation of the viral PR. Analysis of the PR$^-$ virions showed that the amount of SU protein in the Δ873–877 Env mutant was lower than that present on wild-type Env virions despite exhibiting comparable amounts of virion-associated TM protein (Fig. 5B). This result indicates that incorporation of mutant Δ873–877 Env into immature particles does not prevent SU shedding from the virion surface.

Discussion

The available information on the structure of the SIV TM cytoplasmic tail is limited to computer predictions of secondary structure that indicate the presence within this domain of two amphipathic helices which, based on their similarity with natural cytolytic peptides, are designated as LLP-1 and LLP-2 (Miller et al., 1991). Consistent with this prediction, circular dichroism and nuclear magnetic resonance spectroscopy studies have shown that a synthetic peptide derived from LLP-1 forms a helical structure in solution (Yuan et al., 1995). The conservation of the 19 terminal residues of the LLP-1 peptide, not only among the TM proteins of SIV isolates but between SIV and HIV-1 TM proteins as well, indicates that this region may play an important role in regulating Env glycoprotein function.

We therefore decided to investigate whether the introduction of small in-frame deletions within the SIV TM C-terminus affect Env-mediated viral functions. We observed that the Env mutants were synthesized at levels comparable to those of wild-type Env and were processed into the SU and TM subunits. Only a moderate defect in Env processing was evident for mutants Δ882–885 and Leu886stop, which was accompanied by decreased SU shedding from the cell surface. Moreover, all the SIV Env mutants were readily detectable at the cell surface, as judged from the results of the biotinylation experiments, and were found to mediate cell–cell fusion with an efficiency similar to or slightly lower (in the case of the Leu886stop mutant) than that of wild-type Env. However, viruses expressing the mutant Env glycoproteins were poorly infectious in MAGI infectivity assays. When we investigated the cause of this impairment in virus infectivity, we found that although the mutant Env glycoproteins were incorporated into virions as efficiently as wild-type Env, as inferred from the detection of wild-type levels of virion-associated TM protein, the amounts of SU present in mutant virions were significantly lower than those in wild-type particles. This indicates that the mutations engineered into the SIV TM C-terminus weaken the SU-TM interaction, thus promoting SU shedding from the virion surface.

The fact that in the cell context the mutant Env complexes are as stable as those of wild-type Env indicates that the SU-TM instability caused by the Env C-terminal mutations becomes evident only after particle formation. Based on the compelling evidence for a direct interaction between the SIV MA domain and the TM cytoplasmic tail (González et al., 1996; Celma et al., 2001; Manrique et al., 2003), it is conceivable that the stability of the complexes formed by the SU and mutant TM proteins may be more sensitive to the structural rearrangements that Gag undergoes during its assembly into virions than that of the wild-type Env complex.

The SU-TM complex is maintained by noncovalent interactions between the SU and the TM ectodomain (Wyatt et al., 1997). Our results indicate that mutations at the extreme carboxyl end of the SIV TM have long-range effects on the ectodomain of this protein, which alters the strength of the SU-TM association on the virion surface. That mutations in the TM cytoplasmic domain influence Env structure is not without precedent. It has been reported that truncation of the HIV-1 Env cytoplasmic domain as well as certain point mutations within this region modify the sensitivity of gp120 to neutralizing monoclonal and polyclonal antibodies (Edwards et al., 2002; Kalia et al., 2005). Likewise, it has recently been demonstrated for the Moloney murine leukemia virus Env protein that sequential truncations of the TM cytoplasmic tail and membrane-spanning region lead to a differential recognition of the SU subunit by antibodies and also destabilize the SU-TM complex (Aguilar et al., 2003). Our results in SIV thus provide further support to the emerging concept that in retroviruses the TM cytoplasmic domain has an important role in modulating Env structure.

Materials and methods

Cell lines

293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS, GIBCO). MAGI-CCR5 (HeLa-CD4-CCR5/LTR-ß-gal) cells, obtained from the NIH AIDS Research and Reference Reagent Program, were maintained in the same medium as the 293T cells in the presence of 0.2 mg/ml G418 (geneticin), 0.1 mg/ml hygromycin B and 1 μg/ml puromycin.

Env glycoprotein and proviral expression plasmids

Proviral constructs were based on the $SIV_{SMM}PBj1.9$ infectious molecular clone (Dewhurst et al., 1990). Mutagenesis of the TM cytoplasmic domain-coding region was

performed on a *Psh*AI-*Not*I fragment (nucleotides 8170–9996) using the asymmetric PCR-based site-directed mutagenesis method that we have previously described (González et al., 1993). The *Psh*AI-*Not*I fragments carrying the desired mutations were substituted for the wild-type counterpart in the parental DNA construct. To generate the PR^- mutants, the *Bsu*36I-*Bst*XI fragment (nucleotides 2190–3302) in the wild-type and Δ873–877 SIV molecular clones was replaced by the corresponding fragment carrying the D25A/T26S/A28S mutation in the PR-coding region of the *pol* gene that was generated by site-directed mutagenesis as described above. The presence of all the mutations was confirmed by DNA sequencing. The construction of Tat-controlled *env* and *rev* expression vectors was performed as we have recently described (Celma et al., 2004). Briefly, the proviral DNA constructs containing the wild-type or mutated *env* genes were deleted from the *Dra*III to *Spe*I sites (nucleotides 1214 to 3888 in the PBj1.9 genome) to remove the proviral DNA sequences corresponding to the *gag* and *pol* genes. Thus, these vectors contain the reading frames for Env, Tat, and Rev.

Transfections

293T cells (grown in 60-mm-diameter dishes) were transfected with 10 μg of the proviral DNAs or the *env* expression constructs by using Lipofectamine 2000 (Invitrogen) and harvested 48 h posttransfection.

Mutant Env glycoproteins expression

The *env* expression vectors were transfected into 293T cells as described above. 48 h posttransfection, cells were washed twice with ice-cold phosphate-buffered saline solution (PBS) and lysed at 4 °C in lysis buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 10 μg/ml aprotinin), whereas the culture supernatants from the transfected cells were filtered through 0.45-μm-pore-size syringe filters. Cell lysates and culture supernatants were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes and analyzed by Western blotting using the anti-SU MAb KK45 (obtained from the NIH AIDS Research and Reagent Program). Horseradish peroxidase-conjugated anti-mouse immunoglobulin (Amersham Biosciences) was used as secondary antibody. Western blots were developed with an enhanced chemiluminescence and chemifluorescence assay (ECL Plus Reagent, Amersham Biosciences), and the resulting chemifluorescent signal was quantitated as previously described (Manrique et al., 2003).

Cell surface biotinylation

293T cells grown in 35-mm-diameter dishes were transfected with 4 μg of each *env* expression plasmid as described above. At 48 h posttransfection, cells were rinsed three times in ice-cold PBS and were then incubated for 30 min with the membrane-impermeable biotinylating reagent biotinamidocaproate *N*-hydroxysuccinamide ester in 40 mM sodium bicarbonate buffer (pH 8.6) (ECL Protein Biotinylation System; Amersham Biosciences) at 4 °C. The reaction was quenched by washing the cell monolayer twice with ice-cold PBS, and the cells were then lysed in lysis buffer. The cell lysates were immunoprecipitated with pooled sera of infected macaques (kindly provided by Dr. Heeney), and viral proteins were separated on SDS-7.5% polyacrylamide gels and transferred to nitrocellulose membranes. Cell surface proteins were visualized by using streptavidin-conjugated horseradish peroxidase and ECL. To analyze total Env expression, a blot containing a fraction of the immunocomplexes was probed with the anti-SU MAb KK45.

Cell–cell fusion assays

To examine the ability of the mutant Env glycoproteins to mediate cell-to-cell fusion, 293T cells were transiently transfected with each *env* expression construct. 48 h after transfection, the cells were dissociated and equivalent numbers of cells for each Env protein were added, in triplicate, at a 1:10 ratio to 4×10^4 MAGI-CCR5 target cells in 24-well plates. Coculture was continued for 24 h, after which cells were stained for β-galactosidase activity and scored for syncytium formation as described previously (Celma et al., 2004). Syncytia were counted by visual microscopy at 100× magnification. The total number of syncytia (containing five or more nuclei) per field was counted for at least 20 randomly selected fields, and the data were reported as the mean number of blue foci per field.

Single-cycle infectivity assays

Wild-type and mutant SIV proviral constructs were transfected into 293T cells for virus production as described above. At 48 h posttransfection, the supernatants were filtered, normalized for RT activity, and used to infect MAGI-CCR5 cells as we have previously described (Celma et al., 2001; Manrique et al., 2003). At 2 days postinfection, cells were fixed and stained with X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside). Relative infectivity was determined as previously described (Celma et al., 2001; Manrique et al., 2003). Quantitation of virion-associated RT in cell-free culture supernatants from transfected cells was performed by using a nonisotopic RT assay kit (Roche Diagnostics) essentially as previously described (Manrique et al., 2003).

Analysis of Env incorporation into virions

293T cells were transfected with the wild-type and mutant proviral DNAs and 48 h posttransfection cells were washed twice with ice-cold PBS and lysed at 4 °C in lysis buffer. Virions were pelleted from the cell-free culture supernatants by ultracentrifugation (100,000 *g*, 90 min, 4 °C) through a 20% (wt/v) sucrose cushion as we have previously described (Manrique et al., 2003). Cell- and virion-associated proteins

were resolved by SDS-PAGE, blotted onto nitrocellulose membranes, and analyzed by Western blotting using the KK60 MAb to detect the SIV Gag and CA proteins or the KK41 MAb to detect the SIV TM. The KK41 and KK60 MAbs were obtained from J. Stott and K. Kent through the MRC AIDS Directed Program. The SIV SU glycoprotein was detected by using the MAb KK45 obtained from the NIH AIDS Research and Reagent Program.

Acknowledgments

This work was supported by the Fundación Antorchas (Argentina). J.L.A. and S.A.G. are Career Investigators of the National Research Council of Argentina (CONICET).

References

Aguilar, H.C., Anderson, W.F., Cannon, P.M., 2003. Cytoplasmic tail of Moloney murine leukemia virus envelope protein influences the conformation of the extracellular domain: implications for mechanism of action of the R peptide. J. Virol. 77, 1281–1291.

Berlioz-Torrent, C., Shacklett, B.L., Erdtmann, L., Delamarre, L., Bouchaert, I., Sonigo, P., Dokhélar, M.C., Bénarous, R., 1999. Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes modulate intracellular and cell surface expression of envelope glycoproteins. J. Virol. 73, 1350–1361.

Bowers, K., Pelchen-Matthews, A., Honing, S., Vance, P.G., Creary, L., Haggarty, B.S., Romano, J., Ballensiefen, W., Hoxie, J.A., Marsh, M., 2000. The simian immunodeficiency virus envelope glycoprotein contains multiple signals that regulate its cell surface expression and endocytosis. Traffic 1, 661–674.

Celma, C.C.P., Manrique, J.M., Affranchino, J.L., Hunter, E., González, S.A., 2001. Domains in the simian immunodeficiency virus gp41 cytoplasmic tail required for envelope incorporation into particles. Virology 283, 253–261.

Celma, C.C.P., Manrique, J.M., Hunter, E., Affranchino, J.L., González, S.A., 2004. Second-site revertants of a simian immunodeficiency virus gp41 mutant defective in envelope glycoprotein incorporation. AIDS Res. Hum. Retroviruses 20, 733–741.

Chakrabarti, L., Emerman, M., Tiollais, P., Sonigo, P., 1989. The cytoplasmic domain of simian immunodeficiency virus transmembrane protein modulates infectivity. J. Virol. 63, 4395–4403.

Chan, D.C., Kim, P.S, 1998. HIV entry and its inhibition. Cell 93, 681–684.

Chernomordik, L., Chanturiya, A.N., Suss-Toby, E., Nora, E., Zimmerberg, J., 1994. An amphipathic peptide from the C-terminal region of the human immunodeficiency virus envelope glycoprotein causes pore formation in membranes. J. Virol. 68, 7115–7123.

Comardelle, A.M., Norris, C.H., Plymale, D.R., Gatti, P.J., Choi, B., Fermin, C.D., Haislip, A.M., Tencza, S.B., Mietzner, T.A., Montelaro, R.C., Garry, R.F., 1997. A synthetic peptide corresponding to the carboxy terminus of human immunodeficiency virus type 1 transmembrane glycoprotein induces alterations in the ionic permeability of Xenopus laevis oocytes. AIDS Res. Hum. Retroviruses 13, 1525–1532.

Dewhurst, S., Embretson, J.E., Anderson, D.C., Mullins, J.I., Fultz, P.N., 1990. Sequence analysis and acute pathogenicity of molecularly cloned $SIV_{SMM\text{-}PBj14}$. Nature 345, 636–639.

Dubay, J.W., Dubay, S.R., Shin, H.J., Hunter, E., 1995. Analysis of the cleavage site of the human immunodeficiency virus type 1 glycoprotein: requirement of precursor cleavage for glycoprotein incorporation. J. Virol. 69, 4675–4682.

Edwards, T.G., Wyss, S., Reeves, J.D., Zolla-Pazner, S., Hoxie, J.A., Doms, R.W., Baribaud, F., 2002. Truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain of human immunodeficiency virus type 1 envelope glycoprotein. J. Virol. 76, 2683–2691.

González, S.A., Affranchino, J.L., Gelderblom, H.R., Burny, A., 1993. Assembly of the matrix protein of simian immunodeficiency virus into virus-like particles. Virology 194, 548–556.

González, S.A., Burny, A., Affranchino, J.L., 1996. Identification of domains in the simian immunodeficiency virus matrix protein essential for assembly and envelope glycoprotein incorporation. J. Virol. 70, 6384–6389.

Hammonds, J., Chen, X., Ding, L., Fouts, T., De Vico, A., Megede, J.Z., Barnett, S., Spearman, P., 2003. Gp120 stability on HIV-1 virions and Gag-Env pseudovirions is enhanced by an uncleaved Gag core. Virology 314, 636–649.

Hirsch, V.M., Edmonson, P., Murphey-Corb, M., Arbeille, B., Johnson, P.R., Mullins, J.I., 1989. SIV adaptation to human cells. Nature 341, 573–574.

Hunter, E., 1997. gp41, a multifunctional protein involved in HIV entry and pathogenesis. In: Korber, B., Hahn, B., Foley, B., Mellors, J.W., Leitner, T., Myers, G., McCutchan, F., Kuiken, C.L. (Eds.), Human Retroviruses and AIDS 1997: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Los Alamos Natl. Lab, Los Alamos, NM, pp. iii-55–iii-73.

Johnston, P.B., Dubay, J.W., Hunter, E., 1993. Truncations of the simian immunodeficiency virus transmembrane protein confer expanded host range by removing a block to virus entry into cells. J. Virol. 67, 3077–3086.

Kalia, V., Sarkar, S., Gupta, P., Montelaro, R.C., 2005. Antibody neutralization escape mediated by point mutations in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41. J. Virol. 79, 2097–2107.

Kodama, T., Wooley, D.P., Naidu, Y.M., Kestler, H.W., Daniel, M.D., Li, Y., Desrosiers, R.C., 1989. Significance of premature stop codons in env of simian immunodeficiency virus. J. Virol. 63, 4709–4714.

Koenig, B.W., Bergelson, L.D., Gawrisch, K., Ward, J., Ferretti, J.A., 1995. Effect of the conformation of a peptide from gp41 on binding and domain formation in model membranes. Mol. Membr. Biol. 12, 77–82.

LaBranche, C.C., Sauter, M.M., Haggarty, B.S., Vance, P.J., Romano, J., Hart, T.K., Bugelski, P.J., Marsh, M., Hoxie, J.A., 1995. A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. J. Virol. 69, 5217–5227.

Luciw, P.A., Shaw, K.E., Shacklett, B.L., Marthas, M.L., 1998. Importance of the intracytoplasmic domain of the simian immunodeficiency virus (SIV) envelope glycoprotein for pathogenesis. Virology 252, 9–16.

Manrique, J.M., Celma, C.C.P., Affranchino, J.L., Hunter, E., González, S.A., 2001. Small variations in the length of the cytoplasmic domain of the simian immunodeficiency virus transmembrane protein drastically affect envelope incorporation and virus entry. AIDS Res. Hum. Retroviruses 17, 1615–1624.

Manrique, J.M., Celma, C.C.P., Hunter, E., Affranchino, J.L., González, S.A., 2003. Positive and negative modulation of virus infectivity and envelope glycoprotein incorporation into virions by amino acid substitutions at the N-terminus of the simian immunodeficiency virus matrix protein. J. Virol. 77, 10881–10888.

Miller, M.A., Garry, R.F., Jaynes, J.M., Montelaro, R.C., 1991. A structural correlation between lentivirus transmembrane proteins and natural cytolytic peptides. AIDS Res. Hum. Retroviruses 7, 511–519.

Miller, M.A., Cloyd, M.W., Liebmann, J., Rinaldo Jr., C.R., Islam, K.R., Wang, S.Z., Mietzner, T.A., Montelaro, R.C., 1993a. Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. Virology 196, 89–100.

Miller, M.A., Mietzner, T.A., Cloyd, M.W., Robey, W.G., Montelaro, R.C., 1993b. Identification of a calmodulin-binding and inhibitory peptide domain in the HIV-1 transmembrane glycoprotein. AIDS Res. Hum. Retroviruses 9, 1057–1066.

Ritter Jr., G.D., Mulligan, M.J., Lydy, S.L., Compans, R.W., 1993. Cell fusion activity of the simian immunodeficiency virus envelope protein is modulated by the intracytoplasmic domain. Virology 197, 255–264.

Sauter, M.M., Pelchen-Matthews, A., Bron, R., Marsh, M., LaBranche, C.C., Vance, P.G., Romano, J., Haggarty, B.S., Hart, T.K., Lee, W.M., Hoxie, J.A., 1996. An internalization signal in the simian immunodeficiency virus transmembrane protein cytoplasmic domain modulates expression of envelope glycoproteins on the cell surface. J. Cell Biol. 132, 795–811.

Spies, C.P., Compans, R.W., 1994. Effects of cytoplasmic domain length on cell surface expression and syncytium-forming capacity of the simian immunodeficiency virus envelope glycoprotein. Virology 203, 8–19.

Srinivas, S.K., Srinivas, R.V., Anantharamaiah, G.M., Segrest, J.P., Compans, R.W., 1992. Membrane interactions of synthetic peptides corresponding to amphipathic helical segments of the human immunodeficiency virus type-1 envelope glycoprotein. J. Biol. Chem. 267, 7121–7127.

Srinivas, S.K., Srinivas, R.V., Anantharamaiah, G.M., Compans, R.W., Segrest, J.P., 1993. Cytosolic domain of the human immunodeficiency virus envelope glycoproteins binds to calmodulin and inhibits calmodulin-regulated proteins. J. Biol. Chem. 268, 22895–22899.

Tencza, S.B., Mietzner, T.A., Montelaro, R.C., 1997. Calmodulin-binding function of LLP segments from the HIV type 1 transmembrane protein is conserved among natural sequence variants. AIDS Res. Hum. Retroviruses 13, 263–269.

Venable, R.M., Pastor, R.W., Brooks, B.R., Carson, F.W., 1989. Theoretically determined three-dimensional structures for amphipathic segments of the HIV-1 gp41 envelope protein. AIDS Res. Hum. Retroviruses 5, 7–22.

Yuan, T., Mietzner, T.A., Montelaro, R.C., Vogel, H.J., 1995. Characterization of the calmodulin binding domain of SIV transmembrane glycoprotein by NMR and CD spectroscopy. Biochemistry 34, 10690–10696.

Wyatt, R., Desjardin, E., Olshevsky, U., Nixon, C., Binley, J., Olshevsky, V., Sodroski, J., 1997. Analysis of the interaction of the human immunodeficiency virus type 1 gp120 envelope glycoprotein with the gp41 transmembrane glycoprotein. J. Virol. 71, 9722–9731.

Zingler, K., Littman, D.R., 1993. Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein increases Env incorporation into particles and fusogenicity and infectivity. J. Virol. 67, 2824–2831.