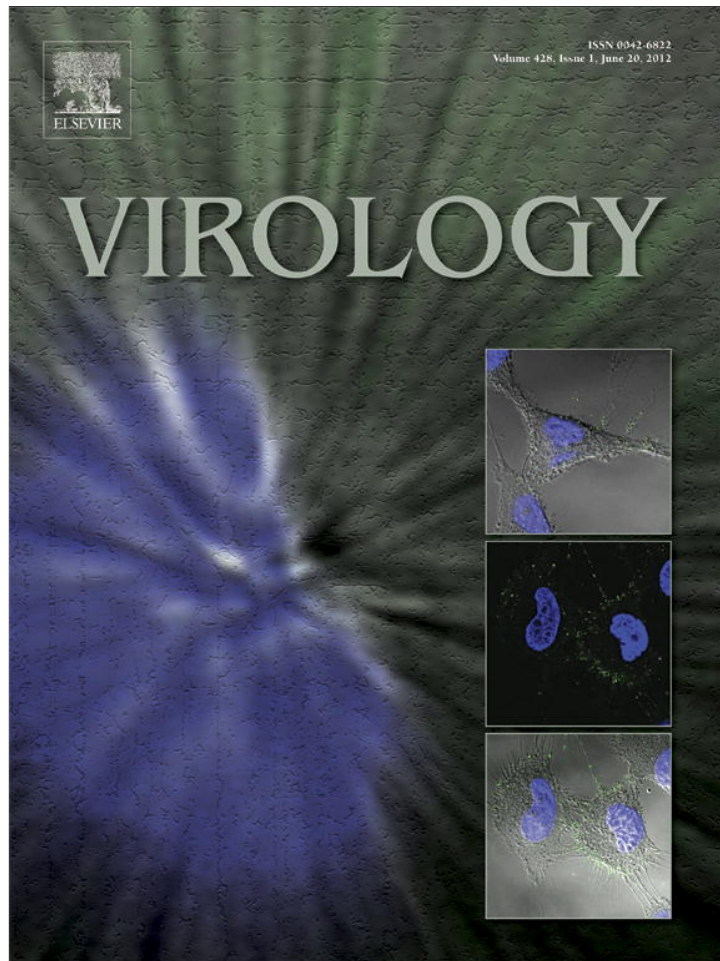


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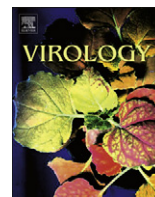
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Palmitoylation of the feline immunodeficiency virus envelope glycoprotein and its effect on fusion activity and envelope incorporation into virions

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ABSTRACT

The feline immunodeficiency virus (FIV) envelope glycoprotein (Env) possesses a short cytoplasmic domain of 53 amino acids containing four highly conserved cysteines at Env positions 804, 811, 815 and 848. Since palmitoylation of transmembrane proteins occurs at or near the membrane anchor, we investigated whether cysteines 804, 811 and 815 are acylated and analyzed the relevance of these residues for Env functions. Replacement of cysteines 804, 811 and 815 individually or in combination by serine residues resulted in Env glycoproteins that were efficiently expressed and processed. However, mutations C804S and C811S reduced Env fusogenicity by 93% and 84%, respectively, compared with wild-type Env. By contrast, mutant C815S exhibited a fusogenic capacity representing 50% of the wild-type value. Remarkably, the double mutation C804S/C811S abrogated both Env fusion activity and Env incorporation into virions. Finally, by means of Click chemistry assays we demonstrated that the four FIV Env cytoplasmic cysteines are palmitoylated.

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Introduction

A number of cellular and viral proteins have been found to be modified by the covalent addition of the 16-carbon saturated fatty acid palmitate (Smotrys and Linder, 2004). Palmitoylation of transmembrane proteins consists in the attachment of palmitic acid via a thioester group to cysteine residues within or at close proximity to the membrane-spanning domain (Resh, 2006; Smotrys and Linder, 2004).

Protein palmitoylation differs from other types of fatty acylation such as prenylation and myristoylation. There is no clear consensus sequence for protein palmitoylation that would allow the identification of potential addition sites by simple examination of the protein primary structure. The most practical approach is to determine if mutagenesis of the cysteine residues at or near the protein membrane anchor affects protein palmitoylation (Drisdell et al., 2006). Another distinctive feature of protein palmitoylation is that it is a reversible process. Indeed, specific enzymes that catalyze protein palmitoylation or depalmitoylation have been identified and characterized in yeast and human cells (see references in Drisdell et al., 2006).

Based on the high hydrophobic character of palmitic acid and on the fact that protein palmitoylation is a dynamic process, several functions have been attributed to this particular kind of fatty

acylation. It has been shown that palmitoylation targets proteins to lipid rafts, specific membrane microdomains that are enriched in cholesterol and glycosphingolipids. This is the case of the influenza virus hemagglutinin which requires three palmitoylated cysteines for efficient association with lipid rafts (Melkonian et al., 1999) and of the luteinizing hormone receptor which localizes to lipid rafts only after palmitate addition to its C-terminus (Lei et al., 2005). Moreover, the reversibility of palmitate attachment to proteins has led to the proposal that this modification plays a critical role in the regulation of protein function and cell signaling, as it has been shown for cell surface receptors and ion channels (Chien et al., 1996; Drisdell et al., 2004).

Retroviruses possess a single envelope glycoprotein (Env), a heterodimer composed of the surface (SU) and transmembrane (TM) subunits that are associated by non-covalent interactions. Several retroviral glycoproteins have been found to be palmitoylated. In both Friend and Moloney murine leukemia viruses (F-MuLV and M-MuLV, respectively) palmitoylation occurs at the cysteine residue within the TM membrane-spanning domain (Hensel et al., 1995; Yang and Compans, 1996). In M-MuLV substitution of this cysteine with serine reduces Env association with lipid rafts, although it should be taken into account that this mutation also impairs Env transport to the cell surface (Li et al., 2002).

Within the membrane anchor region of the Rous sarcoma virus (RSV) TM glycoprotein, the cysteines at positions 164 and 167 are the sites of palmitate addition (Ochsenbauer-Jambor et al., 2001). Replacement of cysteine 167 by glycine results in the formation of poorly infectious virions due to an impairment in Env incorporation into

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particles (Ochsenbauer-Jambor et al., 2001). Interestingly, despite being palmitoylated, the RSV Env glycoprotein does not appear to partition into membrane lipid rafts (Ochsenbauer et al., 2000).

In the case of human and simian immunodeficiency viruses (HIV and SIV, respectively), their TM cytoplasmic domains have also been found to be palmitoylated. However, in contrast to other glycoproteins which are S-acylated at cysteine residues located near or within the membrane anchor, addition of palmitate to the HIV-1 Env protein of the HXB2 strain takes place at cysteines 764 and 837 which are located at a distance of 59 and 132 amino acids, respectively, from the cytoplasmic side of the proposed membrane-spanning domain (Yang et al., 1995). With respect to SIV, palmitic acid has been found to be attached to cysteine 787 in the cytoplasmic domain of the SIVmac239 Env glycoprotein (Yang et al., 1995). Cysteine 787 is at a distance of 71 residues from the C-terminus of the membrane anchor. The fact that HIV-1 and SIV Env proteins are palmitoylated at sites distantly located from the TM membrane-spanning domain may reflect that these primate lentiviral glycoproteins exhibit unusually long cytoplasmic tails, the structure of which has not as yet been resolved. HIV-1 possesses a TM cytoplasmic domain of 150 residues whereas that of SIV is 164 amino acids long.

For HIV-1, partially contradictory results have been reported regarding the effect on Env functions of eliminating the TM palmitoylation sites. Rousso et al. (2000) have shown that removal of both palmitoylation sites in the HIV-1 Env cytoplasmic domain results in decreased Env association with lipid rafts, inhibition of Env incorporation into virions and a 60-fold reduction in viral infectivity. Based on the fact that several infectious HIV-1 molecular clones do not contain cysteines in their Env cytoplasmic domains, two other groups further examined whether Env palmitoylation plays any role in viral infectivity. Using the HIV-1 NL4-3 strain, which conserves the cysteine residue at position 764 but exhibits a tyrosine at position 837, Bhattacharya et al. (2004) have shown that substitution of cysteine 764 and tyrosine 837 by serines or alanines inhibits Env localization in lipid rafts and reduces both Env incorporation and viral infectivity. However, HIV-1 Env mutants carrying at positions 764 and 837 amino acids with bulky hydrophobic side chains, such as phenylalanine and tyrosine, exhibit an infectivity

capacity representing approximately 80% of that of wild-type viruses (Bhattacharya et al., 2004). In this regard, it should be emphasized that those HIV-1 isolates lacking cysteines in their Env cytoplasmic tails bear hydrophobic amino acids with bulky side chains at positions 764 and 837. Surprisingly, Chan et al. (2005) have reported that Cys-to-Ser mutations at HIV-1 Env positions 764 and 837 do not affect either Env cell surface expression or Env incorporation into virions. Moreover, this Env mutant virus efficiently replicates in CD4⁺ T cells (Chan et al., 2005).

Feline immunodeficiency virus (FIV) is a lentivirus that induces in cats a syndrome similar to human AIDS caused by HIV (Pedersen et al., 1987). A distinctive feature of the FIV Env protein is that it exhibits a cytoplasmic domain of 53 amino acids, which is significantly shorter than those of the rest of the lentiviral Env glycoproteins that exceed the size of 100 residues (Verschoor et al., 1993). We have previously shown that the FIV Env cytoplasmic domain plays an important role in regulating Env-mediated viral functions (Celma et al., 2007). Indeed, progressive truncation of the FIV Env cytoplasmic tail from its C-terminus drastically affects either positively or negatively Env fusogenic activity and Env incorporation into virions (Celma et al., 2007).

The cytoplasmic domain of FIV Env has three highly conserved cysteine residues in close proximity to the TM membrane anchor. The disparate data regarding the relevance of Env palmitoylation for HIV-1 replication, prompted us to investigate the role in Env biological properties of the membrane-proximal cysteines within the FIV Env cytoplasmic domain. Furthermore, we examined whether the FIV Env protein is modified by the addition of palmitate.

Results

Mutagenesis of the conserved cysteine residues in the cytoplasmic domain of FIV Env

The cytoplasmic domain of the FIV TM glycoprotein exhibits four cysteines at Env positions 804, 811, 815 and 848 which are highly conserved among viral isolates (Fig. 1). Cysteines 804, 811

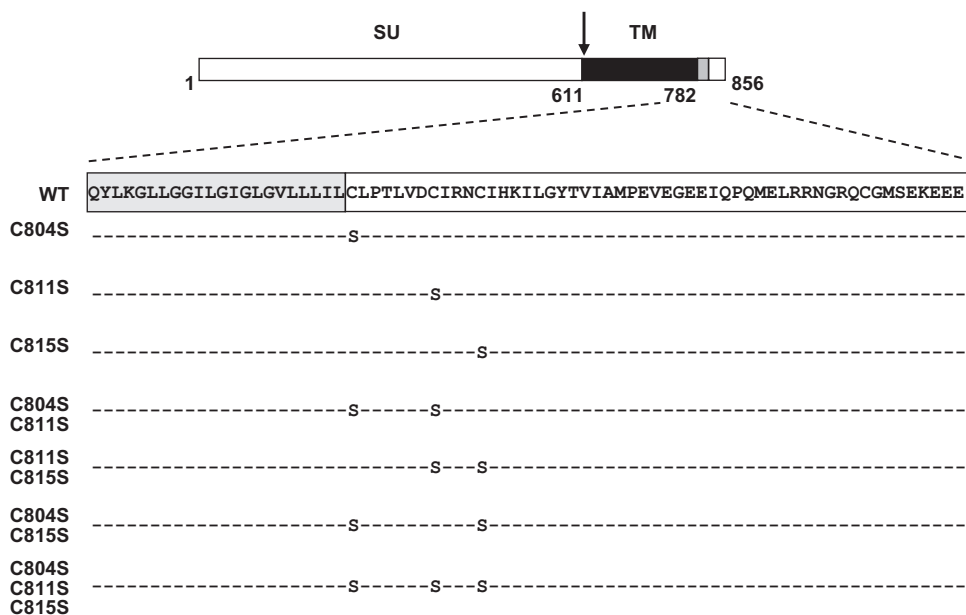


Fig. 1. Schematic diagram of wild-type (WT) and mutant FIV Env proteins. The FIV Env glycoprotein is depicted at the top showing the cleavage site (arrow) between its SU and TM subunits. The TM regions are highlighted: ectodomain (black box), membrane anchor (gray box) and cytoplasmic domain (white box). Amino acid numbering corresponds to the Env protein of the Petaluma F-14 isolate. The different Env mutants used in this study, in which the membrane-proximal cysteines 804, 811 and 815 were replaced alone or in combination by serines, are indicated.

and 815 are located in close proximity to the TM membrane-spanning domain, whereas cysteine 848 is at a distance of only 8 amino acids from the Env C-terminus (Fig. 1). We therefore speculated that, due to their location, cysteines 804, 811 and 815 could be potential sites for protein palmitoylation. To examine the relevance of these membrane-proximal cysteines for Env biological properties, we performed a site-directed mutagenesis study in which we first replaced, alone or in combination, the conserved cysteine residues by serines and then analyzed the effect of these amino acid changes on Env-mediated viral functions. The substitution of a cysteine residue for a serine residue only replaces the thiol group of the former amino acid with a hydroxyl group thereby minimizing the effect of the mutation on the overall protein structure.

We generated three single mutants (C804S, C811S and C815S), two double mutants (C804S/C811S and C811S/C815S) and a triple mutant in which all three cysteines were replaced by serines (Fig. 1).

Synthesis and processing of mutant Env glycoproteins

We first investigated whether mutation of the TM cysteines 804, 811 and 815 affects Env expression or processing. Crandell feline kidney (CrFK) cells were transfected with the wild-type proviral DNA or the FIV genomes carrying the single, double or triple Cys-to-Ser mutations. Immunoblotting of the cell lysates and the clarified supernatants with anti-SU and anti-TM specific antibodies revealed that all mutant Env glycoproteins were expressed and processed as efficiently as wild-type Env (Fig. 2). Indeed, the mutant Env precursors as well as their SU and TM subunits were clearly detected in cell lysates (Fig. 2A and C). An increase in SU shedding with respect to that of wild-type Env was only observed for the C815S and C804S/C811S mutants when the cell-free supernatants were analyzed for the presence of SU (Fig. 2B). Quantitation of the SU levels in the culture supernatants of cells expressing wild-type Env or mutant C815S and

C804S/C811S glycoproteins showed an increase of 1.9 ± 0.1 and 2.4 ± 0.2 for the C815S and C804S/C811S mutants, respectively, when compared to wild-type Env (mean \pm SD, three independent assays). Our results indicate that mutation of TM cysteines 804, 811 or 815 does not significantly affect Env synthesis or processing.

Cell–cell fusion mediated by mutant FIV Env glycoproteins

To evaluate the effect of replacing TM cysteines 804, 811 or 815 by serines on Env fusion activity, we conducted a syncytium formation assay. We have recently developed for the Env protein of the FIV Petaluma isolate a sensitive and quantitative method that assesses fusion between FIV Env-expressing cells and human CXCR4 cells (Celma et al., 2007). 293T cells were cotransfected with the wild-type or TM mutant pcDNAFIV Δ U3 proviral DNAs and the plasmid encoding the HIV-1 Tat protein (see “Materials and methods”). 48 h post-transfection, the cells were dissociated and added to TZM-bl indicator cells. After 48 h of coculture, cells were stained for β -galactosidase activity and scored for syncytia formation. This cell–cell fusion assay is based on the facts that the FIV Petaluma isolate is independent of the CD134 primary receptor for cell entry (Shimojima et al., 2004) and that FIV can efficiently use human CXCR4 (Poeschla and Looney, 1998; Willett et al., 1997).

Under the experimental conditions described above, 293T cells expressing wild-type Env yielded 763 ± 69 blue syncytia per well (average of three independent experiments, \pm SD). When we examined the fusion ability of the mutant Env glycoproteins we found that mutations C804S and C811S significantly impaired Env fusogenicity. Indeed, mutant Env proteins C804S and C811S exhibited fusogenic capacities representing only 16% and 7.6%, respectively, of the wild-type value (Table 1). By contrast, mutation C815S allowed substantial Env fusion activity; mutant C815S retained 50% fusogenic capacity when compared to wild-type Env (Table 1). The most drastic effect on cell–cell fusion was observed for the double (C804S/C811S and C811S/C815S) and triple (C804S/C811S/C815S) mutants.

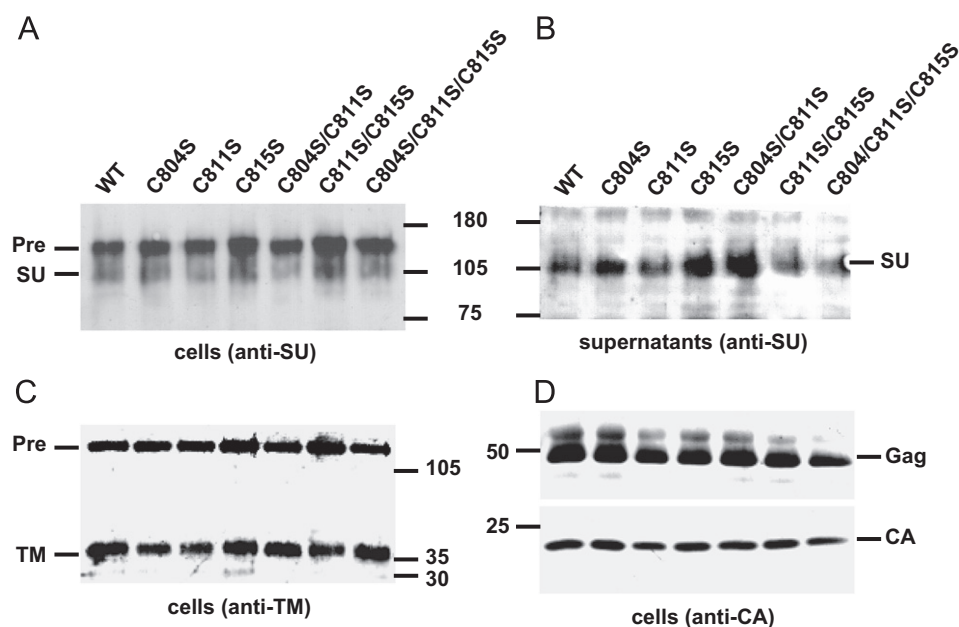


Fig. 2. Synthesis and processing of the FIV Env mutants. CrFK cells were transfected with the wild-type (WT) or mutant proviral DNAs. At 48 h post-transfection, viral proteins from cell lysates (cells) and culture supernatants were subjected to Western blotting using different antibodies. Cell lysates were probed with a MAb directed to the FIV SU (A; anti-SU), a polyclonal serum against the FIV TM (C; anti-TM) or a MAb specific for the FIV capsid protein (D; anti-CA). The supernatants were reacted with the anti-SU MAb (B). The mobilities of the Env precursor (Pre), SU and TM subunits are indicated as are those of the Gag and capsid (CA) proteins. The positions of the molecular weight standards (in kDa) are also included.

Table 1
Cell-to-cell fusion mediated by FIV Env mutant glycoproteins.^a

Env glycoprotein	Relative fusion (% wild-type) ^b
Wild-type	100
Wild-type + AMD3100 (50 µg/ml)	0.3 ± 0.1
C804S	16.2 ± 2.7
C811S	7.6 ± 1.4
C815S	52.2 ± 7.0
C804S/C811S	0.7 ± 0.3
C811S/C815S	3.9 ± 0.7
C804S/C811S/C815S	0.3 ± 0.1

^a 293T cells expressing the wild-type or the mutant FIV Env glycoproteins together with HIV-1 Tat protein were dissociated and equivalent numbers of cells were added at 1:5 ratio to 4×10^4 TZM-bl cells. Coculture was continued for 48 h, after which cells were stained for β -galactosidase and scored for syncytia formation. A fraction of the transfected 293T cells was subjected to immunoblotting to monitor Env expression.

^b Data presented were obtained from three independent experiments, and each assay was performed in triplicate. Blue foci were quantitated as described in "Materials and methods". Values represent means \pm standard deviations.

These amino acid substitutions almost abrogated Env fusogenicity (Table 1). It should be mentioned that the fusion assay used in these experiments is specific for the Env–CXCR4 interaction since addition of the CXCR4 antagonist AMD3100 (50 µg/ml) almost completely inhibits wild-type Env fusion activity (Table 1). Our results therefore demonstrate that, although mutation of any of the TM cysteines at positions 804, 811 and 815 reduces Env-mediated membrane fusion, replacement of cysteines 804 and 811 by serine is highly detrimental to this Env property.

Cell surface expression of FIV Env mutants

Since mutations C804S C811S severely inhibited Env fusogenicity, we decided to examine whether this defect was secondary to an impairment in Env transport to the plasma membrane. We therefore determined the steady-state levels on the cell surface of all the mutant Env glycoproteins and compared them with those of wild-type FIV Env using cellular enzyme-linked immunosorbent assay (cellular ELISA). This technique is highly sensitive and specific for the quantitative analysis of molecules expressed on the cell surface (Józefowski et al., 2011; Lourenço and Roque-Barreira, 2010). 293T cells expressing the wild-type or mutant Env glycoproteins were incubated first with the anti-SU monoclonal antibody (MAb) and then with a horseradish peroxidase (HRP)-coupled secondary antibody as described in "Materials and methods". As shown in Fig. 3A and B, the Env mutants C804S, C811S, C804S/C811S and C804S/C811S/C815S were present at the plasma membrane at levels ranging from 92% to 101% of those of wild-type Env, whereas mutant C811S/C815S attained cell surface levels representing 125% of the wild-type value. By contrast, mutation C815S caused a 24% reduction in Env cell surface expression (Fig. 3A and B).

When Env expression was assessed by ELISA after fixation and permeabilization of the transfected cells, we found that all Cys-to-Ser TM mutants showed levels of total Env protein similar to or even higher than those of wild-type Env (Fig. 3C).

To corroborate that cellular ELISA only quantifies cell surface molecules (Józefowski et al., 2011; Lourenço and Roque-Barreira, 2010), we applied this methodology to 293T cells using as primary antibody a MAb specific for α -tubulin as explained in "Materials and methods". As expected, the absorbance obtained with this primary antibody was similar to the background values obtained with cells incubated only with the secondary antibody. By contrast, α -tubulin was readily detected and quantitated when the cellular ELISA was performed with fixed and permeabilized

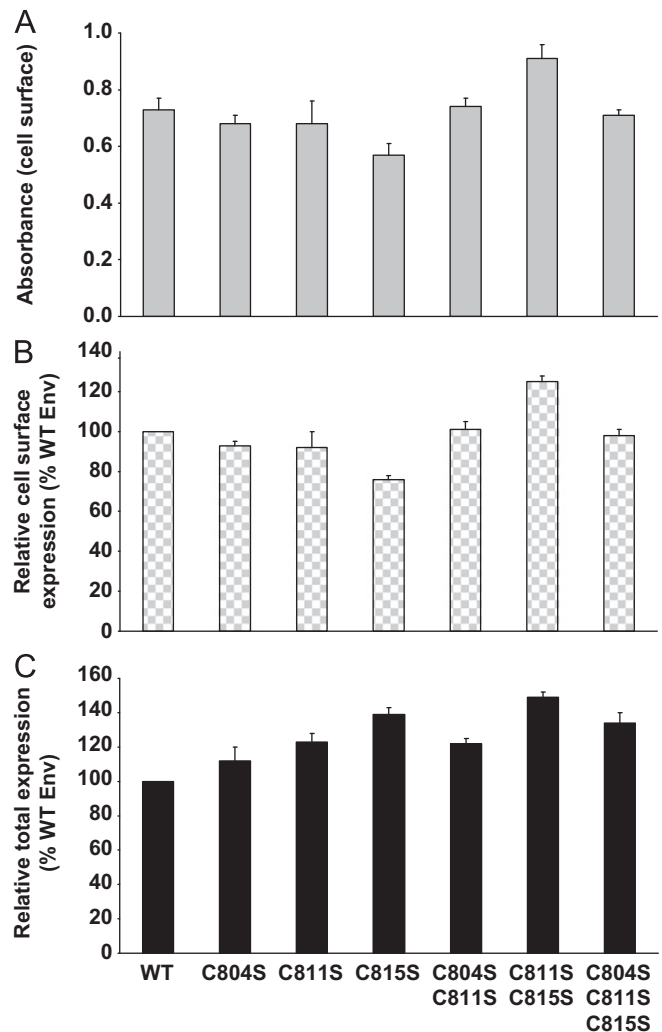


Fig. 3. Cell surface expression of FIV Env mutants. (A) The surface levels in 293T cells of the wild-type and mutant Env glycoproteins were quantitated by cellular ELISA as described in "Materials and methods". The values (mean \pm SD, three independent analyses) correspond to the absorbance of each sample at 405 nm from which the absorbance of non-transfected cells incubated only with the secondary antibody was subtracted. (B) Relative cell surface levels of mutant Env glycoproteins expressed as percentages of those of wild-type Env (considered as 100%). Data were calculated from the values shown in panel A. (C) Cells transfected with the wild-type or the mutant proviral DNAs were fixed and permeabilized and total FIV Env glycoprotein levels were determined by cellular ELISA. Values represent percentages of that of wild-type Env (considered as 100%). Data correspond to the mean \pm SD of three independent experiments.

cells. Indeed, the A_{405} for α -tubulin in intact cells was 0.09 ± 0.02 while that in permeabilized cells was 0.84 ± 0.09 (mean of three independent experiments \pm SD).

In conclusion, our results demonstrate that the FIV Env mutant glycoproteins are as efficiently expressed on the cell surface as wild-type Env. Only in the case of the C815S mutant, a modest reduction in the levels of surface-associated glycoprotein is observed.

Incorporation of the FIV Env mutants into virions

We next examined whether the Cys-to-Ser substitutions introduced into the TM cytoplasmic domain affect Env incorporation into virions. CrFK cells were transfected with the wild-type, C804S, C811S, C815S, C804S/C811S or C804S/C811S/C815S proviral DNAs and virions were then purified from the cell culture

supernatants. The viral proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), blotted and probed for the TM and capsid (CA) proteins as described in "Materials and methods". To evaluate the ability of each mutant Env glycoprotein to associate with particles, virions were tested for the presence of the TM protein instead of the SU subunit because the latter can be spontaneously shed from the cell surface of the transfected cells and therefore contaminate the particulate fraction (Celma et al., 2001). As shown in Fig. 4A, replacement of cysteines 804, 811 or 815 by a serine residue did not affect Env packaging into virions. By contrast, the Env glycoproteins carrying either the double mutation C804S/C811S or the triple substitution C804S/C811S/C815S were not incorporated into particles (Fig. 4B). Indeed, the TM proteins of these mutants could not be detected in the particulate fraction despite the fact that the levels of virion-associated CA protein observed for these mutant viruses were similar to those of their wild-type counterpart (Fig. 4A and B).

Biotinylation experiments of CrFK cells expressing the wild-type Env or the mutant glycoproteins C804S, C811S, C815S and C804S/C811S/C815S demonstrated that the levels of cell surface-associated Env precursor and SU proteins detected for the single and triple TM mutants were comparable to those observed for wild-type Env (Fig. 4C). Similar results were obtained when we applied cellular ELISA to assess the cell surface expression in CrFK cells of the single, double and triple Cys-to-Ser mutants (data not shown). Taken together, these experiments indicate that the defect in Env incorporation exhibited by the double and triple mutants (Fig. 4B) cannot be attributed to inefficient expression of these mutant glycoproteins at the plasma membrane.

Palmitoylation of the FIV TM cytoplasmic domain

Covalent addition of the 16-carbon saturated fatty acid palmitate is a post-translational modification that affects the localization and function of numerous cellular and viral proteins (Resh, 2006). Palmitate is attached through a thioester bond to protein cysteine residues that are at or near the membrane-spanning domain (Resh, 2006). This feature of this particular type of protein acylation led us to speculate that FIV TM cysteines 804, 811 and 815, which are proximal to the membrane-spanning region, could be potential sites of protein palmitoylation.

We therefore first examined whether the cytoplasmic domain of the FIV TM is modified by the addition of palmitate. To this end, we applied a recently developed method that, based on the Click chemistry technology, allows the detection of palmitoylated proteins with high sensitivity and specificity (Charron et al., 2009; Martin and Cravatt, 2009). CrFK cells were transfected with the wild-type FIV proviral DNA and metabolically labeled with azide-conjugated palmitic acid. Cells were lysed and the FIV proteins were immunoprecipitated, eluted from the immunocomplexes and reacted with biotin-alkyne. As negative control, a sample of immunoprecipitated FIV proteins derived from metabolically labeled cells was not incubated with biotin-alkyne. The viral proteins were then resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Fig. 5 shows that the FIV TM is modified by palmitoylation as inferred from its detection using HRP-conjugated streptavidin. The specificity of the reaction is demonstrated by the fact that the TM is visualized only in the protein sample that was incubated with biotin-alkyne (Fig. 5). Moreover, treatment of metabolically labeled samples with hydroxylamine

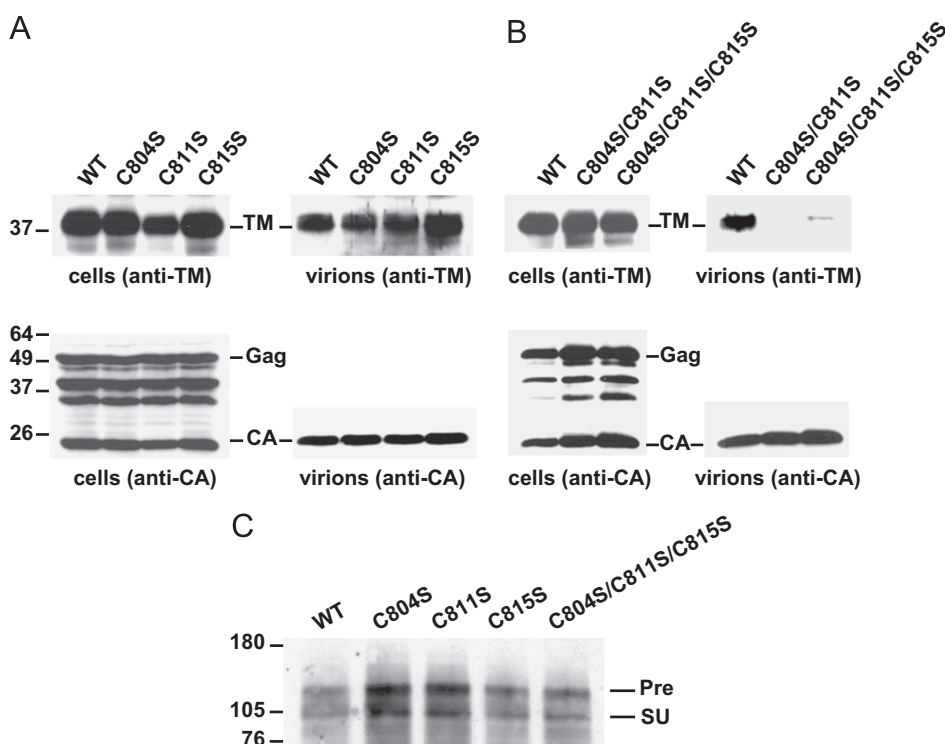


Fig. 4. Incorporation of the FIV Env mutants into virions. CrFK cells were transfected in parallel with the wild-type (WT) proviral DNA and either the single mutant molecular clones (A) or the double and triple mutant proviral DNAs (B). 48 h post-transfection, cells were lysed and the extracellular virions were purified from the clarified culture supernatants by ultracentrifugation through a sucrose cushion as described in "Materials and methods". Viral proteins in the cell lysates and virions were detected by Western blotting using the polyclonal TM antiserum or the MAb directed to the CA protein. The mobilities of the TM and CA proteins are indicated as are those of the molecular weight standards. (C) Cell surface expression of the FIV Env mutants. CrFK cells transfected with the proviral DNAs of wild-type FIV (WT) or of mutants C804S, C811S, C815S and C804S/C811S/C815S were biotinylated as described in "Materials and methods". Env proteins were immunoprecipitated from cell lysates with pooled sera from FIV-infected cats, resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Proteins on the cell surface were detected using streptavidin-conjugated horseradish peroxidase coupled to enhanced chemiluminescence. The mobilities of the Env precursor (Pre) and SU proteins are indicated as are the positions of the molecular weight standards.

(1 M; pH 7.5) before the immunoprecipitation step abrogates TM detection with streptavidin (data not shown).

Localization of the palmitoylation sites in the FIV TM

To identify the sites at which the cytoplasmic domain of the FIV TM glycoprotein is modified by the attachment of palmitic acid, we performed metabolic labeling with palmitic acid-azide of cells transfected with the proviral DNAs of the double mutants C804S/C811S, C811S/C815S and C804S/C815S (the latter mutant was constructed for the purpose of this experiment; Fig. 1). As negative control, we included the triple mutant C804S/C811S/C815S. The rationale behind this experiment was that with the aid of the FIV Env double mutants we would be able to identify which cysteines are palmitoylated. Surprisingly, the triple mutant

in which cysteines 804, 811 and 815 are replaced by serines, was found to be modified by palmitoylation (Fig. 6A). This result suggested that the distal cysteine residue at Env position 848 might be also modified by the addition of palmitate.

We therefore created a mutant proviral DNA in which the four cysteines within the TM cytoplasmic domain were replaced by serines. When we compared the results of the metabolic labeling with palmitic acid-azide of cells expressing the triple and quadruple Env cysteine mutants with those obtained with wild-type Env, we found that Env palmitoylation is abrogated only after simultaneously substituting the four cysteine residues within the FIV TM cytoplasmic tail (Fig. 6B).

Taken together, these data indicate that in the FIV TM protein palmitic acid is attached not only to the membrane-proximal cysteine residues, but to the distal cysteine 848 as well.

Triton X-100 solubility properties of wild-type and mutant FIV Env glycoproteins

The results obtained at this point indicated that while both C804S and C804S/C811S/C815S mutations were highly detrimental to Env fusion activity, the mutant C804S Env protein was efficiently incorporated into virions whereas the triple mutant glycoprotein was incapable of associating with particles. Taking into account that it has been demonstrated in HIV-1 that a substantial fraction of Gag is associated with lipid rafts (Lindwasser and Resh, 2001; Nguyen and Hildreth, 2000; Ono and Freed, 2001), we decided to examine whether the triple substitution C804S/C811S/C815S impairs Env localization in detergent-resistant membrane microdomains thereby preventing Env packaging into Gag-made particles.

One property of lipid raft-associated proteins is their insolubility in Triton X-100 at 4 °C (Brown and Rose, 1992). We therefore transfected 293T cells with the wild-type, C804S and C804S/C811S/C815S proviral DNAs and subjected the cells to two

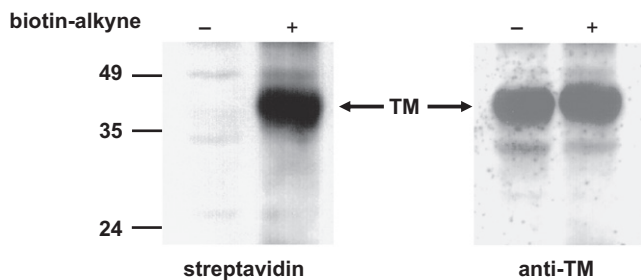


Fig. 5. Palmitoylation of the FIV TM cytoplasmic domain. CrFK cells were transfected with the wild-type FIV proviral DNA, metabolically labeled with palmitic acid-azide, lysed and immunoprecipitated as described in "Materials and methods". Viral proteins were then subjected to Click chemistry either in the presence (+) or in the absence (-) of biotin-alkyne, resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was first incubated with streptavidin-horseradish peroxidase conjugate (left panel) and then reprobbed with the anti-TM polyclonal serum (right panel). The mobility of the FIV TM protein is indicated.

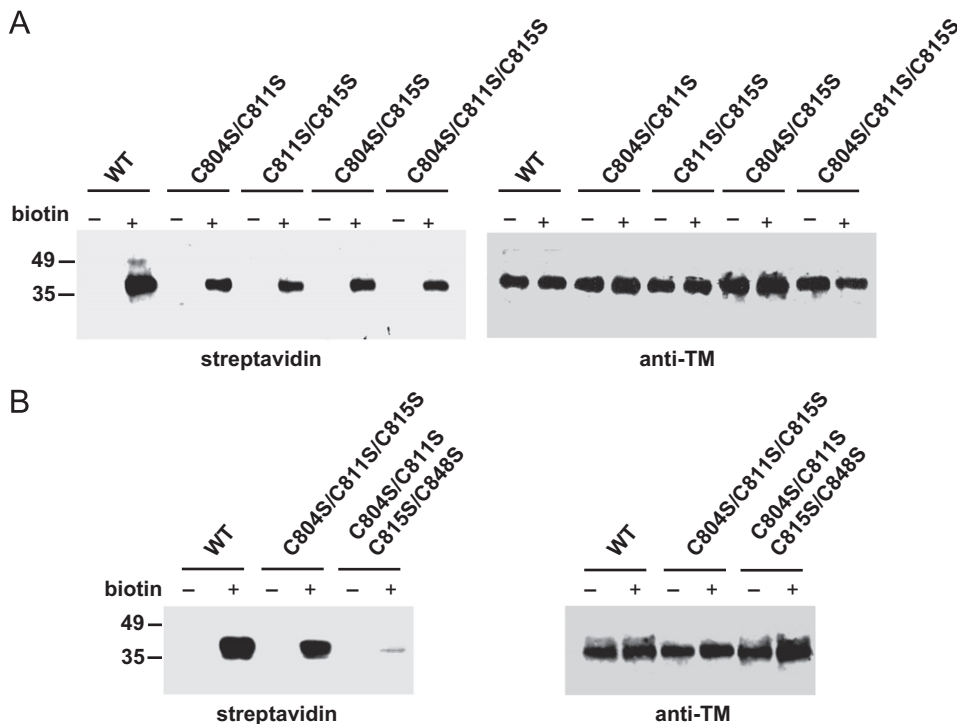


Fig. 6. Identification of the palmitoylation sites in the FIV TM cytoplasmic domain. (A) CrFK cells were transfected with the wild-type (WT), C804S/C811S, C811S/815S, C804S/C815S and C804S/C811S/C815S proviral DNAs and metabolically labeled with palmitic acid-azide. Viral proteins were immunoprecipitated from cell lysates and subjected to Click chemistry to detect palmitate addition as described in "Materials and methods". (B) CrFK cells transfected with the wild-type (WT) molecular clone or the mutant C804S/C811S/C815S and C804S/C811S/C815S/C848S proviral DNAs were metabolically labeled with palmitic acid-azide and then processed as described above.

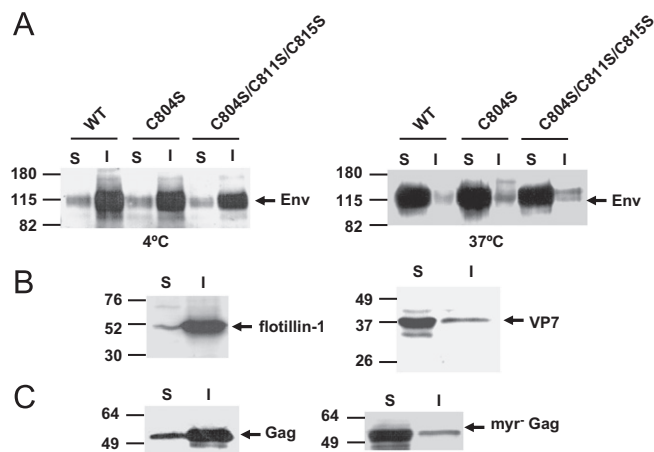


Fig. 7. Triton X-100 solubility of the wild-type and mutant Env glycoproteins. (A) 293T cells expressing wild-type (WT), C804S or C804S/C811S/C815S Env proteins were extracted with Triton X-100 at 4 °C (left panel) or at 37 °C (right panel), and soluble (S) and insoluble (I) fractions were obtained as described in “Materials and methods”. The FIV glycoproteins were detected in each fraction using the anti-SU MAb. (B) 293T cells that constitutively express flotillin-1 as well as 293T cells infected with a recombinant vaccinia virus directing the synthesis of rotavirus VP7 were processed at 4 °C into the Triton X-100 soluble (S) and insoluble (I) fractions and visualized by Western blotting as described in “Materials and methods”. (C) 293T cells were transfected with the wild-type FIV proviral DNA or a molecular clone expressing a nonmyristoylated version of the Gag polyprotein (myr⁻ Gag). Cells were subjected to the Triton X-100 extraction procedure at 4 °C and soluble (S) and insoluble (I) fractions were obtained by centrifugation as described in “Materials and methods”. Both the wild-type and myr⁻ Gag proteins were detected by Western blotting using the MAb directed to the CA domain of Gag.

successive detergent extractions as described in “Materials and methods”. Cells were first lysed in Triton X-100 at 4 °C and then centrifuged so as to obtain the Triton-soluble material in the supernatant. The pellet was resuspended in β -octylglucoside, a detergent that disrupts lipid rafts, and centrifuged to recover the Triton-insoluble material in the supernatant. We found that the wild-type and mutant Env glycoproteins were mostly detected in the Triton X-100 insoluble fractions (Fig. 7A). By contrast, when the Triton X-100 extraction was performed at 37 °C instead of 4 °C, the wild-type and mutant glycoproteins shifted from the insoluble to the soluble fraction (Fig. 7A). Of note, when using the same two-step detergent extraction method, we observed that the lipid raft marker flotillin-1 was recovered in the insoluble fraction, whereas the rough endoplasmic reticulum integral membrane glycoprotein rotavirus VP7 was solubilized by treatment with Triton X-100 at 4 °C (Fig. 7B). Interestingly, most of the FIV Gag protein was detected in the insoluble fraction (Fig. 7C). However, a nonmyristoylated FIV Gag, which we have previously shown to be incapable of plasma membrane binding (Manrique et al., 2001b), was found in the soluble fraction (Fig. 7C). These results indicate that under our experimental conditions the virion incorporation-defective C804S/C811S/C815S mutant Env glycoprotein is as resistant to Triton X-100 extraction at 4 °C as wild-type Env.

Discussion

The cytoplasmic domain of the FIV Env glycoprotein has four highly conserved cysteine residues at positions 804, 811, 815 and 848. Cysteines 804, 811 and 815 are located in close proximity to the TM membrane anchor occupying positions 1, 8 and 12, respectively, in the cytoplasmic tail.

Given that divergent results have been reported with respect to the functions played by the retroviral TM cytoplasmic cysteines and their palmitoylation, we decided to investigate the relevance

of the FIV TM membrane-proximal cysteines 804, 811 and 815 for Env biological properties. To this end, we constructed a series of single, double and triple mutants in which cysteines 804, 811 and 815 were replaced, alone or in combination, by serines. When these FIV Env mutants were phenotypically characterized, we found that they were all expressed and processed into the SU and TM subunits in a wild-type manner. By contrast, when we tested the fusogenic capacity of the TM cysteine mutants, we observed that substitution for serine of cysteines 804 or 811 drastically reduced the ability of these glycoproteins to mediate cell-to-cell fusion when compared with wild-type Env. A highly defective fusion phenotype was also demonstrated for the double mutant C804S/C811S and the triple mutant C804S/C811S/C815S. Interestingly, replacement of cysteine 815 by serine allowed Env fusion to proceed with an efficiency representing approximately 50% of that of wild-type Env. Taken together, our results clearly indicate that cysteines 804 and 811 are critical for FIV Env fusogenicity. It should be stressed that the defect in Env fusion imposed by the C804S and C811S mutations cannot be attributed to an impairment in Env transport to the plasma membrane since these mutant Env glycoproteins are readily detected at the cell surface at levels comparable to those of wild-type Env.

A crucial step in lentiviral morphogenesis is the incorporation of Env into the Gag-made budding virions. In the case of SIV, we have demonstrated by means of genetic, biochemical and pull-down experiments that Env packaging into viral particles results from a specific interaction between the Env cytoplasmic tail and the matrix domain of the Gag protein (Celma et al., 2001; González et al., 1996; Manrique et al., 2001a, 2003, 2008). The same mechanism operates in HIV-1 (Freed and Martin, 1996; Murakami and Freed, 2000). When we examined the ability of the most relevant FIV Env cysteine mutants generated in this study to associate with viral particles, we found that the substitution of cysteines 804, 811 or 815 by serine do not affect Env incorporation into virions. However, the double C804S/C811S and the triple C804S/C811S/C815S mutations abrogate Env packaging into particles. There are at least two possible explanations for the Env incorporation defect exhibited by the double and triple Env mutants. These amino acid substitutions may modify the structure of the FIV TM thereby impairing its association with Gag. Alternatively, the double C804S/C811S and the triple C804S/C811S/C815S mutations may alter the distribution of Env on the plasma membrane which in turn may prevent its recruitment into budding particles. However, the mutant Env glycoprotein lacking the three most membrane-proximal cytoplasmic cysteines is resistant to Triton X-100 extraction at low temperature, suggesting that this protein is associated with lipid rafts. The same behavior is exhibited by both wild-type Env and Gag. It is therefore likely that the triple mutant Env glycoprotein is not excluded from the virus assembly sites at the plasma membrane. The fact that removal of three of the four palmitoylation sites from the FIV Env glycoprotein does not appear to affect its cell surface distribution is in line with the results of Yang et al. (2010) showing that the two cytoplasmic palmitoylated cysteines in the HIV-1 Env protein are not involved in its association with lipid rafts. Instead, HIV-1 Env localization to detergent-resistant membrane domains is promoted by molecular determinants present within the C-terminal α -helix of the cytoplasmic tail (Yang et al., 2010).

We have previously shown that removal of the entire FIV TM cytoplasmic domain or the deletion of as many as six amino acids into the membrane-spanning region blocks Env incorporation into virions (Celma et al., 2007). However, the phenotype of these Env mutants is explained by the instability of the SU-TM complex exhibited by these glycoproteins, which causes SU secretion and TM degradation (Celma et al., 2007). Therefore, mutations C804S/C811S

and C804S/C811S/C815S are the first amino acid substitutions to be described for the FIV TM cytoplasmic domain that abrogate Env incorporation without affecting Env processing or cell surface expression.

An important result stemming from our work is the demonstration, by means of the Click chemistry technology, that the FIV TM cytoplasmic tail is palmitoylated and that the four highly conserved cysteine residues within this Env domain are the sites of this modification. In this regard, the endodomain of the spike glycoprotein of SARS-coronavirus bears nine cysteine residues, all or most of which appear to be palmitoylated (Petit et al., 2007).

Although we expected that the best candidates for palmitoylation would be the membrane-proximal cysteines 804, 811, and 815, we found that the distal cysteine residue at position 848 is acylated as well. Cysteine 848 is at a distance of 44 amino acids from the TM membrane anchor. In this respect, in the Env proteins of most HIV-1 and SIV isolates the TM cytoplasmic cysteines that are modified by palmitate addition are also distantly located from the membrane-spanning domain (Yang et al., 1995). We did not address in this work the role in Env function of cysteine 848 because we have previously characterized an FIV Env truncation mutant lacking the C-terminal 11 amino acids (including cysteine 848) which showed a wild-type phenotype with respect to fusion activity and Env incorporation into virions (Celma et al., 2007).

In summary, we show here that the FIV TM cytoplasmic tail is modified by the addition of palmitate to the four highly conserved cysteine residues present in this Env domain. Moreover, our results indicate that the two most membrane-proximal cysteines (residues 804 and 811) are more essential to FIV functions than cysteines 815 and 848, since mutants C804S and C811S are poorly fusogenic and the simultaneous mutation of these residues not only abrogates Env fusion ability but Env incorporation into virions as well.

Materials and methods

Cell lines

293T, CrFK and TZM-bl cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS, GIBCO).

Monoclonal antibodies

Monoclonal antibodies used to detect FIV SU (SUFc1-30) and CA proteins (PAK3-2C1) were obtained from the NIH Research and Reference Reagent Program. Pooled sera from FIV-infected cats were provided by Edward Hoover (Colorado State University, USA). Detection of FIV TM protein was performed using a mouse polyclonal antibody generated in our laboratory (Celma et al., 2007).

Proviral DNA constructs and site-directed mutagenesis

All FIV Env mutant proviruses were derived from the infectious molecular clone F-14 of the Petaluma isolate (Olmsted et al., 1989). Mutation of the TM cytoplasmic domain was performed by asymmetric PCR-based site-directed mutagenesis as we have previously described using the *Elongase* enzyme high-fidelity mix (Invitrogen) and antisense oligonucleotides carrying the desired mutations (Celma et al., 2007; Manrique et al., 2004). The single, double and triple mutations affecting the TM cysteine residues at Env positions 804, 811 and 815 were introduced into a *SpeI*-*NotI* restriction fragment corresponding to nucleotides (nts)

8287–9474 of the FIV genome. The *SpeI*-*NotI* fragments containing the corresponding mutations were substituted for the wild-type counterpart in the parental FIV construct cloned in the pSV-SPORT1 plasmid (Celma et al., 2007). We have previously generated an FIV proviral DNA lacking the U3 element of the 5' long terminal repeat (LTR) which was cloned immediately downstream of the pcDNA human early cytomegalovirus promoter (Celma et al., 2007). This construct, denominated pcDNAFIVΔU3, allows the expression of the FIV viral proteins in human cells (Celma et al., 2007; Poeschla and Looney, 1998). The pSV-SPORT1 constructs carrying the mutant proviral DNAs were digested with *KpnI* and *NotI*, and the resulting fragments (nts 6398–9474 of the FIV genome) were subcloned into the *KpnI* and *NotI* sites of the pcDNAFIVΔU3.

Transfections and viral protein analysis

CrFK or 293T cells (grown in 60-mm-diameter dishes) were transfected with 8 μg of wild-type or mutant proviral DNAs using Lipofectamine 2000 (Invitrogen). 48 h post-transfection cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed at 4 °C in buffer containing 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 followed by a 5-min centrifugation at 16,000g to remove cellular debris. Culture supernatants from the transfected cells were filtered through 0.45-μm-pore-size filters (Corning). Cell lysates and clarified supernatants were resolved by SDS-PAGE, blotted onto nitrocellulose membranes and analyzed by Western blotting using the MAbs directed to the SU and CA proteins or the anti-TM polyclonal serum. Western blots were developed with ECL Advance reagent (GE Life Sciences). Protein band signals were quantitated as previously described (Celma et al., 2007; Rauddi et al., 2011).

Analysis of Env incorporation into virions

Virions were pelleted from the cell-free culture supernatants of transfected CrFK cells by ultracentrifugation (100,000g, 90 min, 4 °C) through a 20% (w/v) sucrose cushion as we have previously described (Celma et al., 2007; Manrique et al., 2004). Virion-associated proteins were resolved by SDS-PAGE and analyzed by Western blotting using the PAK3-2C1 MAb to visualize the Gag and CA proteins, and the mouse anti-TM serum to detect the FIV TM.

Cellular ELISA

To determine the cell surface levels of FIV Env, 293T or CrFK cells were transfected with the wild-type or mutant proviral DNAs as described above. 48 h post-transfection, triplicate samples corresponding to 3×10^5 cells were first washed with ice-cold PBS and resuspended in 50 μl of ELISA buffer (PBS containing 0.4% bovine serum albumin [BSA] and 0.1% sodium azide). Cells were incubated with 10 μg/ml anti-SU SUFc1-30 MAb for 1 h at 4 °C, washed 3 times with ice-cold ELISA buffer and exposed to 10 μg/ml HRP-conjugated anti-mouse IgG (Promega) for 1 h at 4 °C. Cells were washed 3 times with ELISA buffer and the enzymatic reaction was performed using the 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate. The resulting colored reaction signal was measured on a microtiter plate ELISA reader (Biorad) at 405 nm (reference wavelength 490 nm) as we have previously described (Rauddi et al., 2011). Non-transfected cells incubated with the secondary antibody were used as a negative control. Moreover, to corroborate that under our experimental conditions only cell surface proteins are detected, we carried out the cellular ELISA protocol described above using a

MAB against the intracellular α -tubulin protein as primary antibody (Sigma-Aldrich).

To quantitate total cellular Env expression, 10^5 transfected cells were fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, rinsed 3 times in PBS and permeabilized with 0.1% (v/v) Triton X-100 for 15 min at room temperature. After washing the cells 3 times with PBS, they were resuspended in PBS containing 0.4% BSA and successively incubated with the anti-SU MAB and the HRP-conjugated anti-mouse antibody. Enzyme activity was measured in an ELISA reader as described above.

Cell surface biotinylation

Biotinylation of surface proteins in transfected cells was performed essentially as we have previously reported (Affranchino and González, 2006; Celma et al., 2007). Transfected CrFK cells were incubated for 30 min at 4 °C with the membrane-impermeable biotinylating reagent biotinamidocaproate N-hydroxy-succinamide ester (ECL Protein Biotinylation System, GE Life Sciences). Cells were washed twice with ice-cold PBS and lysed as described above. Viral proteins were immunoprecipitated with pooled sera from FIV-infected cats, resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Cell surface proteins were detected by HRP-conjugated streptavidin and ECL.

Cell–cell fusion assays

To investigate the fusogenic capacity of the mutant FIV Env proteins we performed a cell-to-cell fusion assay that we have recently developed (Celma et al., 2007). Briefly, 293T cells were transfected with the wild-type and mutant pcDNAFIV Δ U3 constructs together with an HIV-1 Tat-expressing plasmid created in our laboratory (Celma et al., 2007). 48 h post-transfection, the cells were dissociated and equivalent number of cells were added at a 1:5 ratio to 4×10^4 TZM-bl cells in 24-well plates. TZM-bl cells are HeLa derivatives that express high levels of CD4 and both CXCR4 and CCR5 coreceptors and carry the β -galactosidase gene under the control of the HIV-1 LTR (Wei et al., 2002). Coculture of transfected 293T and indicator TZM-bl cells was continued for 48 h after which cells were stained for β -galactosidase activity and scored for syncytia formation as previously described (Celma et al., 2007). Cell-to-cell fusion was quantitated as the total number of blue syncytia per well by first counting their number in at least 20 nonoverlapping fields. The average number of syncytia per field was multiplied by the total number of fields per well and the result was referred to that obtained for wild-type Env. In the case of Env mutants yielding low number of syncytia, the entire wells were examined and the total number of blue syncytia was counted. The CXCR4 antagonist AMD3100 was purchased from Sigma-Aldrich.

Metabolic labeling with palmitic acid and Click chemistry

To examine whether the cytoplasmic domain of the FIV TM is modified by the addition of palmitic acid we performed metabolic labeling of FIV Env-expressing cells with palmitic acid-azide followed by detection of its incorporation into the TM protein using Click chemistry (Charron et al., 2009; Martin and Cravatt, 2009). Namely, CrFK cells (grown in 35-mm-diameter dishes) were transfected in duplicate with wild-type or mutant proviral DNAs. At 48 h post-transfection, cells were incubated during 1 h in FBS-free DMEM after which cells were metabolically labeled with 100 μ M palmitic acid-azide (Invitrogen) for 6 h in DMEM supplemented with 2.5% (v/v) FBS. Cells were washed twice with ice-cold PBS and lysed as described above. Cell lysates were

immunoprecipitated with pooled sera from FIV-infected cats and immunocomplexes were eluted from the protein A-Sepharose beads by incubation at 80 °C during 15 min in buffer containing 50 mM Tris-HCl (pH 8.0), 1% SDS, 1 mM PMSF and 10 μ g/ml aprotinin. The eluates containing the FIV proteins were subjected to Click chemistry using the Click-iT Metabolic Labeling Reagents for Proteins (Invitrogen). The viral proteins obtained from one set of transfections were incubated with 20 μ M biotin-alkyne (Invitrogen) in the presence of 2 mM CuSO₄ as catalyst during 20 min at room temperature. The reaction was stopped by the addition of 3 volumes methanol, 0.75 volumes chloroform and 2 volumes water and centrifuged at 16,000g during 5 min. Proteins present in the interface layer were precipitated by the addition of methanol, centrifuged at 16,000g during 5 min. The protein pellets were air-dried and resuspended in non-reducing loading buffer. The other set of protein samples was treated likewise except that the biotin-alkyne reaction step was omitted. All samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were first incubated with HRP-conjugated streptavidin to visualize the palmitoylated proteins and then probed with the mouse polyclonal anti-TM serum.

Triton X-100 solubilization assay

To evaluate the solubility in Triton X-100 of wild-type and mutant Env glycoproteins we followed a protocol similar to that described by Adam et al. (2008) but with several modifications. 293T cells were transfected with the wild-type, C804S or C804S/C811S/C815S proviral DNAs, and 48 h post-transfection cells were washed twice with ice-cold PBS and lysed on ice for 5 min in a solution containing 0.2% Triton X-100, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 50 mM KCl, 10 mM MgCl₂, 10 mM EGTA, 5 mM dithiothreitol and protease inhibitors. Cell lysates were then centrifuged at 16,000g for 60 min at 4 °C. The supernatants containing the detergent-soluble fractions were separated from the pellets corresponding to the Triton X-100 insoluble material. The pellets were resuspended in a lysis solution with a composition similar to that described above but containing 60 mM β -octylglucoside instead of Triton X-100. The samples were incubated on ice for 10 min and centrifuged as described above. The two sets of supernatants resulting from the two-step detergent extraction method were immunoprecipitated with pooled sera from FIV-infected cats, subjected to SDS-PAGE and transferred to nitrocellulose membranes. FIV Env proteins were detected by Western blotting using the anti-SU MAB whereas FIV Gag was visualized with the MAB directed to the CA domain. Non-transfected 293T cells were subjected to the same two-step detergent extraction assay described above and the lipid raft-associated protein flotillin-1 was visualized in the resulting supernatants with a MAB (BD Biosciences) kindly provided by Eduardo Castaño (Leloir Institute, Argentina). Rotavirus VP7 (SA11 strain) was expressed in 293T cells using a recombinant vaccinia virus as we have previously described (Celma et al., 2001) and detected by means of an anti-SA11 polyclonal rabbit serum. To express nonmyristoylated FIV Gag, we transfected 293T cells with a proviral DNA in which the codon for the glycine residue at position 2 in the gag open reading frame was mutated to alanine (Manrique et al., 2001b).

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