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Importance of the short cytoplasmic domain of the feline immunodeficiency virus transmembrane glycoprotein for fusion activity and envelope glycoprotein incorporation into virions

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Abstract

The mature form of the envelope (Env) glycoprotein of lentiviruses is a heterodimer composed of the surface (SU) and transmembrane (TM) subunits. Feline immunodeficiency virus (FIV) possesses a TM glycoprotein with a cytoplasmic tail of approximately 53 amino acids which is unusually short compared with that of the other lentiviral glycoproteins (more than 100 residues). To investigate the relevance of the FIV TM cytoplasmic domain to Env-mediated viral functions, we characterized the biological properties of a series of Env glycoproteins progressively shortened from the carboxyl terminus. All the mutant Env proteins were efficiently expressed in feline cells and processed into the SU and TM subunits. Deletion of 5 or 11 amino acids from the TM C-terminus did not significantly affect Env surface expression, fusogenic activity or Env incorporation into virions, whereas removal of 17 or 23 residues impaired Env-mediated cell-to-cell fusion. Further truncation of the FIV TM by 29 residues resulted in an Env glycoprotein that was poorly expressed at the cell surface, exhibited only 20% of the wild-type Env fusogenic capacity and was inefficiently incorporated into virions. Remarkably, deletion of the TM C-terminal 35 or 41 amino acids restored or even enhanced Env biological functions. Indeed, these mutant Env glycoproteins bearing cytoplasmic domains of 18 or 12 amino acids were found to be significantly more fusogenic than the wild-type Env and were efficiently incorporated into virions. Interestingly, truncation of the TM cytoplasmic domain to only 6 amino acids did not affect Env incorporation into virions but abrogated Env fusogenicity. Finally, removal of the entire TM cytoplasmic tail or deletion of as many as 6 amino acids into the membrane-spanning domain led to a complete loss of Env functions. Our results demonstrate that despite its relatively short length, the FIV TM cytoplasmic domain plays an important role in modulating Env-mediated viral functions.

Keywords: Feline immunodeficiency virus; Envelope glycoprotein; Transmembrane glycoprotein; Envelope cytoplasmic domain; Fusogenic activity

Introduction

Feline immunodeficiency virus (FIV) is a lentivirus that induces in cats a progressive disease similar to human AIDS caused by human immunodeficiency virus (HIV) (Pedersen et al., 1987). FIV is therefore intensively studied not only because it constitutes an important health problem for domestic cats but also as a useful model for HIV-1 infections in humans. FIV infects a broad range of cell types such as $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes and macrophages (Brown et al., 1991; Brunner and Pedersen, 1989; Dean et al., 1999; Dow et al., 1999; English et al., 1993). Entry of FIV into their target cells is initiated by the specific interaction of the viral envelope glycoprotein (Env) with cellular receptors. In contrast to HIV-1, FIV does not utilize the CD4 molecule as primary receptor (Hosie et al., 1993). However, the FIV Env protein binds to the chemokine receptor CXCR4 (Poeschla and Looney, 1998) which is used as coreceptor by T-tropic HIV-1 strains (Richardson et al., 1999; Willett et al., 1997). Furthermore, productive infection of feline $CD4^+$ T cells requires, in addition to virus interaction with CXCR4, Env attachment to the recently identified cell surface receptor CD134 (de Parseval et al., 2004;

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Shimojima et al., 2004). Thus, by mediating virus entry, FIV Env plays a key role in determining viral tropism and pathogenesis.

FIV Env, like that of other lentiviruses, is synthesized as a heavily glycosylated precursor which is cleaved by cellular proteases into the surface (SU) and transmembrane (TM) subunits which remain associated by non-covalent interactions. Translation of the FIV *env* open reading frame yields a protein product of approximately 150 kDa which is processed into a 130-kDa species by removal of an unusually long N-terminal signal peptide (Verschoor et al., 1993). The 130-kDa Env molecule is then further processed into two polypeptides of 95 kDa and 36 kDa which correspond to the SU and TM glycoproteins, respectively (Verschoor et al., 1993). Evidence has been presented indicating that the SU and TM subunits of FIV Env perform similar functions to those of their HIV-1 counterparts. Indeed, the FIV SU appears to mediate binding to the cell surface receptors (de Parseval and Elder, 2001), whereas the FIV TM mediates fusion of the viral membrane with that of the target cell (Garg et al., 2004; Giannecchini et al., 2004).

The TM subunit of lentiviral Env glycoproteins shares a conserved structural organization: an ectodomain containing a hydrophobic fusion peptide, a single membrane-spanning domain and a C-terminal cytoplasmic region (Hunter, 1997). A characteristic feature of most lentiviral TM proteins is the presence of a cytoplasmic tail of 100–200 amino acids, whose length contrasts with that (30–40 amino acids) of other retroviral Env proteins (Hunter and Swanstrom, 1990). The biological function of the long cytoplasmic domain of lentiviral Env proteins has been the subject of several studies essentially focused on HIV-1 and simian immunodeficiency virus (SIV). In this regard, mutations in the Env cytoplasmic tail of these viruses have been shown to affect viral functions, such as regulation of Env expression at the cell surface (Berlioz-Torrent et al., 1999; LaBranche et al., 1995), syncytium formation (Earl et al., 1991; Gabuzda et al., 1992; Ritter et al., 1993) and virus infectivity (Chakrabarti et al., 1989; Dubay et al., 1992). Moreover, we and others have demonstrated that domains within the C-terminal third of the HIV-1 and SIV Env cytoplasmic tail are responsible for Env glycoprotein incorporation into virions by establishing specific interactions with the matrix (MA) domain of the viral Gag polyprotein (Celma et al., 2001; Freed and Martin, 1995, 1996; González et al., 1996; Kalia et al., 2003; Mammano et al., 1995; Piller et al., 2000; Manrique et al., 2001, 2003; Murakami and Freed, 2000; Yu et al., 1993). Taken together, these studies highlight the important role played by the TM cytoplasmic domain in Env-mediated viral functions. However, compared with the amount of information available for the cytoplasmic regions of HIV-1 and SIV Env proteins, little is known about the function of the equivalent region in other lentiviruses. In particular, the analysis of the FIV TM cytoplasmic region seems relevant since it is unusually short (about 50 amino acids) when compared not only with that of primate lentiviruses (150 and 164 residues for HIV-1 and SIV, respectively) but also with that of lentiviruses of ungulate animals, such as caprine arthritis and encephalitis virus (129 residues), equine infectious anemia virus (200 residues) and visna virus (126 residues) (Rice et al., 1990; Saltarelli et al., 1990; Sonigo et al., 1985).

Given the importance of the Env glycoprotein in the lentiviral life cycle, in the present report we investigated the role that the FIV TM cytoplasmic domain plays in modulating Env structure and function. We studied how variations in the length of the Env cytoplasmic region affect the synthesis, processing, cell surface expression, incorporation into virions and fusogenic capacity of the FIV Env glycoprotein.

Results

Construction of FIV Env mutants carrying premature stop codon mutations in the TM cytoplasmic region

Based on computer-assisted programs that predict protein secondary structure and hydrophobicity, it has been proposed that the membrane-spanning domain of the FIV TM protein comprises Env residues 786–812 which would result in a TM cytoplasmic domain of 53 amino acids (Verschoor et al., 1993). However, the boundaries of the FIV Env membrane-spanning domain and the length of the TM cytoplasmic region need to be experimentally confirmed. To study the biological functions of the FIV Env cytoplasmic domain, we performed a mutagenesis analysis of the C-terminal 59 amino acids of Env. We engineered into the *env* gene of the infectious molecular clone FIV-14 of the Petaluma isolate a series of premature stop codon mutations so as to progressively truncate the TM C-terminus by 5, 11, 17, 23, 29, 35, 41, 47, 53 or 59 amino acids (Fig. 1). Among these Env constructs, mutants TM198, TM192 and TM186 were designed to help define the cytoplasmic border of the Env membrane-spanning domain (Fig. 1).

Synthesis and processing of the FIV Env glycoproteins bearing truncated cytoplasmic domains

We first investigated whether the progressive truncation of the FIV TM cytoplasmic region had any effect on Env synthesis and processing. To this end, wild-type and Env mutant proviral DNAs were transfected in parallel into Crandell feline kidney (CrFK) cells and 48 h post-transfection both the cell lysates and the clarified cell culture supernatants were analyzed by Western blotting (Fig. 2). Immunoblotting of the cell lysates with an anti-SU monoclonal antibody (MAb) revealed that all the mutant FIV Env proteins were expressed as efficiently as wild-type Env, as inferred from the detection of wild-type levels of the mutant Env proteins (Fig. 2A). When the cell-associated viral proteins were resolved on 7.5% polyacrylamide gels and probed with an anti-TM polyclonal serum, it was observed that the mutant Env precursor glycoproteins exhibited an increased electrophoretic mobility with respect to the wild-type Env, which is in agreement with the expected molecular weight reduction resulting from the premature stop codon mutations introduced into the *env* gene (Fig. 2A). Furthermore, all the mutant Env proteins were found to be processed since the SU glycoprotein was detected in both the cell lysates and cell

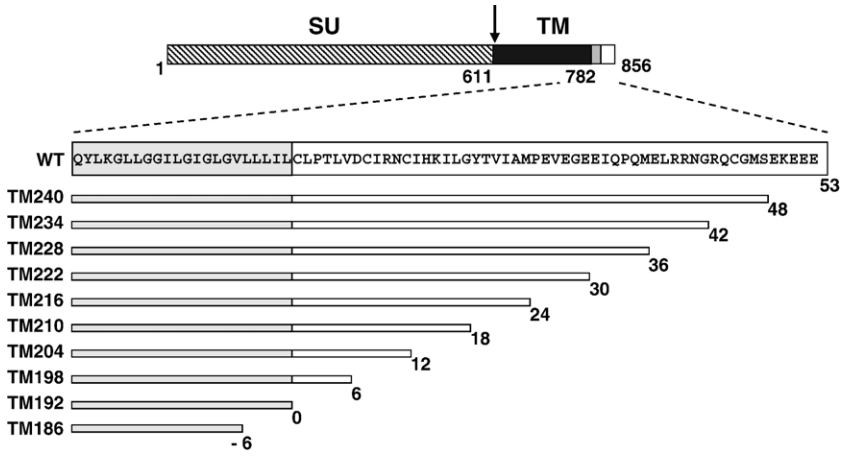

Fig. 1. Construction of the FIV Env truncation mutants. The wild-type (WT) FIV Env glycoprotein is depicted at the top showing the cleavage site between its SU and TM subunits (arrow). The structural features of the TM glycoprotein are shown: ectodomain (black box), membrane-spanning domain (gray box) and cytoplasmic region (open box). Amino acid numbering corresponds to the Env protein of the Petaluma FIV-14 isolate. The Env truncation mutants are named according to the length of their TM subunits, whereas the numbers at the right of each construct refer to the length of the cytoplasmic domain resulting from the introduction of the stop codon mutations in the *env* gene.

culture supernatants (Fig. 2A). Quantitation of the intracellular and extracellular levels of the Env glycoproteins showed that mutants TM240, TM234, TM228, TM222, and TM210 were processed with an efficiency similar to or slightly lower than that of wild-type Env, whereas the degree of mutant TM216 processing was less than 50% of the wild-type value (Fig. 2B). By contrast, the Env mutants bearing a cytoplasmic domain of 12 or 6 amino acids were found to be processed more efficiently than wild-type Env, exhibiting processing indices of 1.53 (mutant TM204) and 1.37 (mutant TM198) (Fig. 2B). Likewise, removal of the entire TM cytoplasmic domain (mutant TM192) as well as C-terminal truncation of the TM glycoprotein by as many as 6 amino acids into the putative membrane-spanning domain (mutant TM186) also led to enhanced Env processing (Fig. 2B). In addition, mutations TM204 to TM186 reduced the stability of the SU-TM association with respect to that of wild-type Env. Indeed, these mutants exhibited association indices ranging from 0.49 to 0.62 (Fig. 2B), together with enhanced shedding of the SU glycoprotein (Fig. 2A). Of note, when the cell lysates were probed with a polyclonal serum directed against the TM ectodomain, we could not detect the bands corresponding to the TM subunits of mutants TM192 and TM186 (Fig. 2C). This suggests that either both mutant Env precursors are not stably anchored in the plasma membrane or that after Env processing, the TM subunits of these mutants are rapidly degraded. However, the fact that neither the Env precursors of mutants TM192 and TM186 nor their TM subunits could be detected in the cell culture supernatants by using the anti-TM serum (data not shown) favors the latter possibility.

It should be mentioned that mutations TM240 and TM234 that introduce nucleotide changes into the Rev responsive element (nucleotides 8785–8928 in the FIV genome; Phillips et al., 1992) do not disrupt its function, since wild-type levels of Gag (Fig. 2D) and Env proteins (Fig. 2A) were detected in cells expressing these TM mutants.

Cell surface expression of the FIV Env mutants

To examine whether progressive truncation of the FIV TM cytoplasmic domain affected the transport of the Env proteins to the plasma membrane, we performed cell surface biotinylation of CrFK cells transfected in parallel with the wild-type or mutant FIV proviral DNAs. In the case of the wild-type FIV Env, surface biotinylation resulted mainly in the detection of the Env precursor (Fig. 3). The difficulty in detecting the FIV SU glycoprotein at the cell surface may be related to the low levels of intracellular SU protein detected in transfected cells (Fig. 2A; Pancino et al., 1995; Verschoor et al., 1993). Comparison of the cell surface levels of the wild-type FIV Env precursor with those of the mutant Env proteins revealed that all mutant Env glycoproteins were transported to the plasma membrane, albeit with different efficiencies (Fig. 3A). Mutants TM240, TM234, TM228, TM210 and TM204 were present at the cell surface at levels comparable to those of wild-type Env (Fig. 3B). A moderate defect in cellular transport was caused by mutations TM222, TM198 and TM192. Indeed, these mutants exhibited levels of surface-associated Env precursor ranging from 51% to 60% of those of wild-type Env (Fig. 3B). The most drastic effect on Env surface expression was observed for mutants TM216 and TM186 whose levels at the cell membrane were found to be reduced by approximately 72% and 84%, respectively, when compared to those of wild-type Env.

Fusogenic capacity of the FIV TM mutants

Following the interaction of the SU subunit of the FIV Env with the receptor–coreceptor complex present at the surface of the target cells, the TM glycoprotein mediates fusion of the viral and cellular membranes, thereby allowing virus entry. Thus, Env function can be assessed by evaluating its ability to mediate cell–cell fusion. To analyze the fusogenic capacity of the FIV Petaluma Env protein, we developed a sensitive and consistent

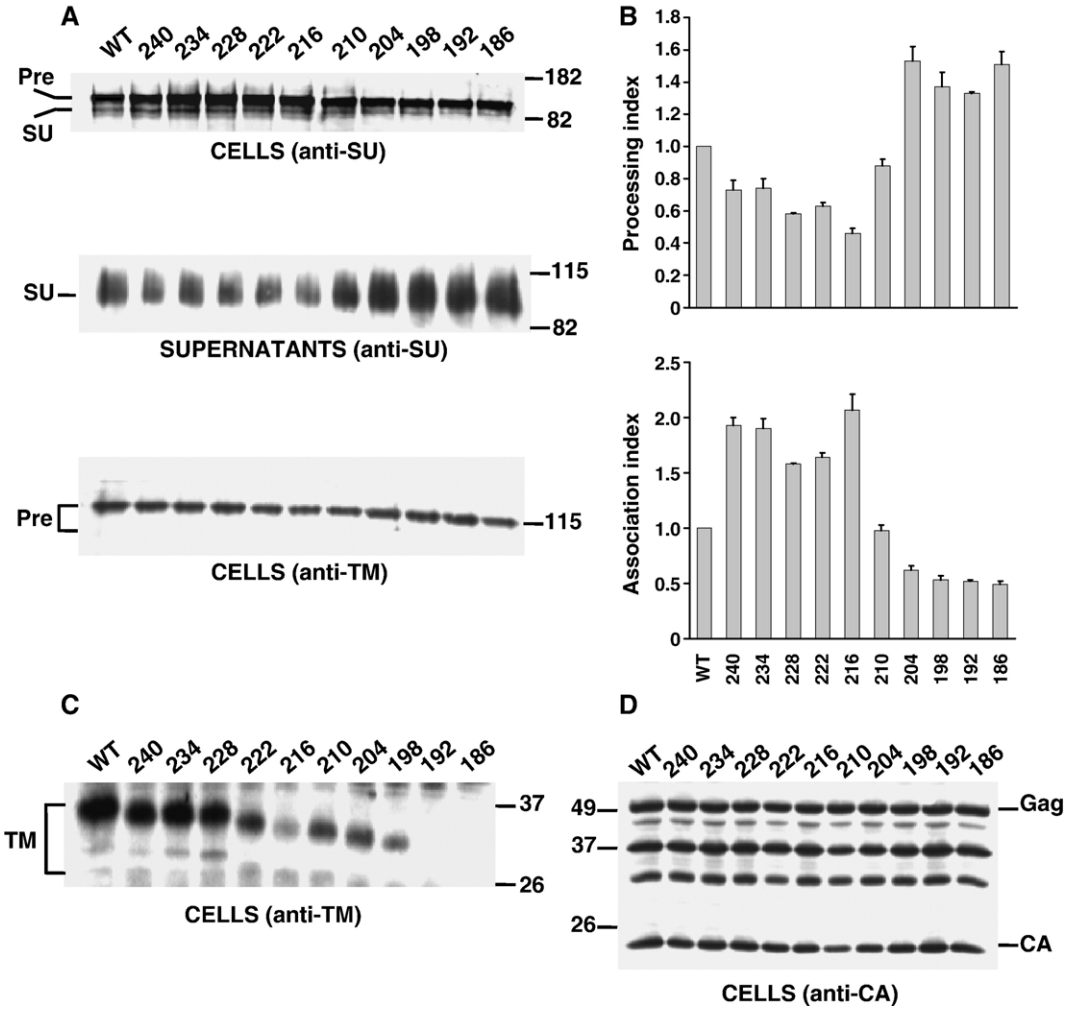

Fig. 2. Synthesis and processing of the FIV Env truncation mutants. CrFK cells were transfected with the wild-type (WT) or TM mutant FIV proviral clones. At 48 h post-transfection, viral proteins from cell lysates (CELLS) and culture supernatants (SUPERNATANTS) were transferred to nitrocellulose membranes and subjected to Western blotting using different antibodies. (A) Immunoblotting of cell lysates (10% polyacrylamide gel) and culture supernatants (7.5% polyacrylamide gel) with a MAb directed to the FIV SU glycoprotein (anti-SU). Cell lysates (resolved on 7.5% polyacrylamide gel) were also probed with a polyclonal serum against the FIV TM protein (anti-TM). The mobilities of the FIV 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 TM mutants in the cell lysates and culture supernatants were quantitated 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. The data presented are the mean values and standard deviations of three independent assays. (C) Immunoblotting of cell lysates using an anti-TM polyclonal serum. Proteins were resolved on a 11% polyacrylamide gel. The mobilities of the wild-type and truncated TM proteins are shown. (D) Probing of cell lysates with a MAb specific for the FIV capsid (CA) protein. The mobilities of the Gag and CA proteins are indicated.

approach that assays fusion between FIV Env-expressing cells and human CXCR4 cells (see Materials and methods). 293T cells were cotransfected with the pcDNA-ΔU3FIV proviral DNA, an FIV DNA construct that allows viral proteins to be efficiently expressed in human cells, and a plasmid encoding the HIV-1 Tat protein. Forty-eight hours after transfection, the cells were dissociated and added to MAGI-CXCR4 indicator cells (HeLa-CD4-LTR-β-galactosidase; Kimpton and Emerman, 1992). Coculture was continued for 48 h after which cells were stained for β-galactosidase activity and scored for syncytia formation. The choice of the MAGI cell line as the target indicator cells in this assay was based on the fact that cell fusion mediated by the Petaluma Env protein is only CXCR4-dependent and does not require the CD134 cellular receptor (Shimojima et al., 2004). Under these experimental conditions, 293T cells expressing wild-type FIV Env yielded 1077±178 blue syncytia per well (average of four assays±SD). By contrast, mixing of MAGI-CXCR4 cells with 293T cells expressing HIV-1 Tat alone did not yield any blue cells. When the fusogenic capacity of the FIV TM mutants was evaluated, we found that removal of the C-terminal 5 or 11 amino acids from the TM cytoplasmic domain (mutations TM240 and TM234) did not affect Env-mediated cell-to-cell fusion (Table 1). Further truncation of the FIV Env C-terminus by 17, 23 or 29 amino acids (mutations TM228, TM222, and TM216) increasingly inhibited Env fusogenic capacity. Indeed,

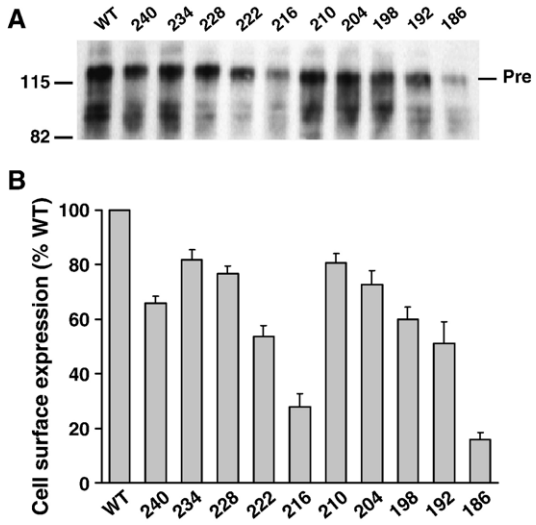

Fig. 3. Cell surface expression of the FIV Env truncation mutants. CrFK cells were transfected with the wild-type (WT) or TM mutant FIV proviral DNAs and biotinylated as described in Materials and methods. (A) Env proteins were immunoprecipitated from cell lysates, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Proteins on the cell surface were visualized by using streptavidin–horseradish peroxidase conjugate coupled with enhanced chemiluminescence. The mobility of the Env precursor protein is shown. (B) Levels of surface-associated Env precursor for each TM mutant relative to those of wild-type Env.

mutant TM216 mediated cell-to-cell fusion with an efficiency representing only 20% of the wild-type value (Table 1). Interestingly, FIV Env proteins bearing a cytoplasmic domain of 18 or 12 amino acids (mutantsTM210 and TM204) were found to be significantly more fusogenic (approximately 1.5-fold) than the wild-type Env. A drastic change in the level of fusion was observed when the length of the TM cytoplasmic domain was shortened from 12 amino acids (mutant TM204) to 6 residues (mutant TM198). The latter mutation resulted in a reduction of fusion by 94% with respect to the wild-type value (Table 1). Moreover, removal of the C-terminal 53 amino acids of the FIV Env glycoprotein (mutant TM192) as well as further truncation of the TM protein by 6 residues into the membrane-spanning domain (mutant TM186) abolished Env fusogenicity (Table 1). Our results indicate that progressive truncation of the FIV TM cytoplasmic domain severely affects Env fusogenic capacity.

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

Env glycoprotein	Relative fusion (% wild-type)[b]
Wild-type	100
TM240	93.2±7.1
TM234	113.0±6.4
TM228	53.6±8.7
TM222	37.1±6.9
TM216	20.4±3.4
TM210	140.5±13.1
TM204	135.5±8.3
TM198	6.0±2.5
TM192	ND[c]
TM186	ND[c]

[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 MAGI-CXCR4 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 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.

[c] No blue syncytia were detected.

Effect of progressive truncation of the FIV TM cytoplasmic domain on Env incorporation into virions

We next investigated the ability of the mutant Env glycoproteins to be incorporated into virions. To this end, CrFK cells were transfected with either the wild-type or the mutant proviral DNAs, and 48 h post-transfection virions were purified by ultracentrifugation from the clarified cell culture supernatants. Virion lysates were analyzed for the presence of viral proteins by Western blotting. The process of Env incorporation into virions was first examined by analyzing the levels of virion-associated SU protein (Fig. 4A). Most of the mutant Env glycoproteins (mutants TM240 to TM222 and mutants TM210 to TM198) were incorporated into virions at levels similar to those of wild-type Env. By contrast, the SU glycoprotein of Env mutants TM216, TM192 and TM186 was not detected on virions, indicating that these mutations cause a drastic defect in Env incorporation.

Further analysis of virion samples with the anti-TM serum (Fig. 4B) paralleled the results obtained with the anti-SU MAb. Indeed, virion-associated TM protein was readily detected for mutants TM240 to TM222 and for mutants TM210 to TM198,

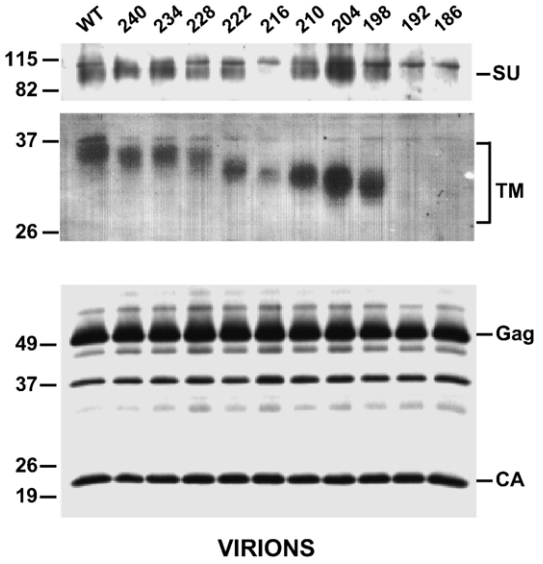

Fig. 4. Incorporation of truncated Env glycoproteins into virions. CrFK cells were transfected with the wild-type (WT) or TM mutant proviral DNAs. At 48 h post-transfection, virions were purified and viral proteins were analyzed by Western blotting using antibodies specific for the FIV SU, TM or CA proteins. The mobilities of the SU, TM, Gag and CA proteins are shown. The positions of the molecular weight standards are indicated on the left.

whereas no TM protein was found in virions purified from cells transfected with the mutant TM192 and TM186 proviral constructs. In the case of mutant TM216, the TM glycoprotein could be detected on virions, albeit at levels representing approximately 10% of the wild-type value. Of note, the mutant TM204 Env glycoprotein was incorporated into virions more efficiently than the wild-type Env, exhibiting levels of virion-associated SU and TM proteins (relative to those of capsid protein) corresponding to 149.7±5.2% and 139.5±10.7%, respectively, of those found in wild-type virions. This indicates that truncation of the TM cytoplasmic domain to 12 amino acids enhances FIV Env incorporation into virions.

Discussion

FIV is unique among lentiviruses since it exhibits an Env glycoprotein with a putative cytoplasmic domain of 53 amino acids which is significantly shorter than that of the rest of the lentiviral glycoproteins that range in size from 100 to 200 residues. Based on this particular feature of the FIV Env glycoprotein, in this paper we addressed whether variations in the length of the TM cytoplasmic tail had any effect on Env-mediated viral functions. We inserted by site-directed mutagenesis a series of stop codon mutations into the coding region for the C-terminal domain of the FIV TM in order to generate Env mutants bearing progressively truncated cytoplasmic tails.

All the mutant Env glycoproteins were expressed in feline cells at levels comparable to those of the wild-type Env and were normally processed into the SU and TM subunits. However, increased shedding of the SU glycoprotein into the cell culture supernatants was observed for those mutants bearing cytoplasmic tails of 12 or 6 amino acids (mutants TM204 and TM198), indicating that these mutations reduce the stability of the SU-TM association. A similar phenotype was exhibited by mutant TM192 in which the entire Env cytoplasmic tail was removed and by mutant TM186 which bears a large C-terminal deletion extending by 6 amino acids into the membrane-spanning domain. Moreover, we found that in the case of mutants TM192 and TM186 the TM subunit of Env could not be detected in transfected feline cells by Western blotting. The fact that the SU subunits of these Env mutants were readily detected in both cell lysates and cell culture supernatants indicates that the Env precursors of mutants TM192 and TM186 are transported through the secretory pathway and efficiently processed but upon Env cleavage, the SU-TM complex becomes unstable which in turn leads to the release of the SU subunit and degradation of the TM glycoprotein. This also explains the complete loss of membrane fusion activity and lack of Env incorporation into virions observed for mutants TM192 and TM186 (see below).

The presence of biologically functional FIV Env complexes on the cell surface was investigated by fusion assays using human cells expressing the mutant Env glycoproteins and MAGI-CXCR4 indicator cells, an approach that provided a highly quantitative measure of the Env syncytium-forming ability. Removal of the C-terminal 5 or 11 amino acids from the FIV TM did not significantly affect Env fusogenicity. By contrast, further truncation of the TM cytoplasmic tail by 17, 23 or 29 residues impaired Env-mediated cell-to-cell fusion by 47%, 63% and 80%, respectively, compared to the wild-type Env fusogenic capacity. Remarkably, when 35 or 41 amino acids were deleted from the TM C-terminus (mutants TM210 and TM204), the resulting Env glycoproteins were more fusogenic than the wild-type Env. Noteworthy, these mutant Env glycoproteins, which bear cytoplasmic tails of 18 or 12 amino acids, lack the GYTVL motif (TM residues 211–214) which is analogous to the tyrosine-containing endocytosis motif GYXXÖ present in the cytoplasmic domains of HIV-1 and SIV Env proteins (Hosie et al., 2005). Although our biotinylation experiments did not allow us to unequivocally detect the surface-associated SU glycoprotein of these Env mutants, it is tempting to speculate that the enhanced fusogenic capacity of mutants TM210 and TM204 with respect to wild-type Env may be related to increased levels of biologically active SU glycoprotein at the cell surface due to a reduced rate of mutant protein endocytosis. Alternatively, shortening the FIV TM cytoplasmic tail to 18 or 12 residues may facilitate Env mobility on the membrane thereby promoting Env clustering during membrane fusion. Another possibility is that removal of 35 or 41 amino acids from the TM C-terminus may cause structural changes in the TM ectodomain, favoring the necessary folding transitions into the fusion-active state which would result in increased fusion efficiency or kinetics. In support of the notion that the TM cytoplasmic domain plays an important role in modulating Env structure, it has been shown that deletions at the TM C-terminus affect the fusion kinetics of HIV-1 Env (Wyss et al., 2005), the exposure of HIV-1 SU epitopes (Edwards et al., 2002) and the strength of the SU-TM association on SIV virions (Affranchino and González, 2006).

It is interesting to compare the phenotype of mutants TM210 and TM204, which were found to be more fusogenic than wild-type Env, with that of mutant TM198. The latter Env mutant, whose cytoplasmic domain is only 6 amino acids shorter than that of mutant TM204, is highly inefficient at mediating cell-to-cell fusion. Indeed, mutant TM198 exhibits only 6% of the wild-type Env syncytium-forming ability. Similarly, removal of the entire cytoplasmic tail (mutant TM192) or deletion of as many as 6 amino acids into the membrane-spanning domain (mutant TM186) abrogates Env fusogenicity. Taken together, our results indicate that the FIV TM cytoplasmic tail tolerates only a moderate reduction in its length without losing its full fusogenic ability. However, truncation of the TM cytoplasmic region to 18 or 12 amino acids results in Env glycoproteins with more fusogenic activity than the wild-type Env. In this regard, it has been well documented that in the primate lentiviruses HIV-1 and SIV, certain properties of the Env glycoproteins such as membrane fusion and cell tropism can also be augmented by truncating their cytoplasmic tails to 18 amino acids (Johnston et al., 1993; Spies and Compans, 1994; Zingler and Littman, 1993). It is therefore interesting to conclude that, irrespective of the original size of the TM cytoplasmic tail, a length of 18 amino acids for this region appears to be optimal for the biological activities displayed by diverse lentiviral Env proteins. However, there is a strong selection pressure to maintain

in vivo an Env glycoprotein with an intact cytoplasmic domain (Kodama et al., 1989). This probably reflects, as we have previously proposed for SIV Env (Manrique et al., 2001), the presence at the Env C-terminus of molecular determinants required for full virus pathogenicity or a viral replication strategy that requires Env expression and function to be kept tightly regulated.

A critical step in the late stages of the lentiviral life cycle is the incorporation of the Env glycoprotein into virions. For HIV-1 and SIV, we and others have demonstrated that this process is mediated by specific interactions between the TM cytoplasmic tail and the MA domain of Gag (Celma et al., 2001; Freed and Martin, 1996; Manrique et al., 2003; Murakami and Freed, 2000; Wyma et al., 2000). The requirement in primate lentiviruses of specific protein–protein interactions for Env incorporation reflects the complexity of the mechanism by which the Gag-made particles accommodate an Env glycoprotein with a long cytoplasmic tail. It was therefore interesting to investigate whether in FIV the integrity of the relatively short TM cytoplasmic region was also necessary for Env incorporation into particles. Our mutagenesis studies show that most of the truncated FIV Env glycoproteins were efficiently packaged into virions. The sole exceptions were Env mutants TM216, TM192 and TM186. In the case of the defect in Env incorporation observed for mutant TM216, it is probably related to the inefficient expression of this mutant on the cell surface as inferred from the results of the biotinylation experiments, whereas for mutants TM192 and TM186, their inability to be packaged into particles most likely reflects the instability of the SU-TM complex after Env processing which causes SU secretion and TM degradation. In marked contrast, mutant TM204 was found to be incorporated into virions at levels significantly higher than those of wild-type Env. Therefore, abbreviation of the FIV TM cytoplasmic tail to 12 amino acids not only enhances Env fusogenicity but increases Env incorporation levels as well. At least two explanations that are not mutually exclusive may account for the increased ability of mutant TM204 to be packaged into particles. Truncation of the FIV TM cytoplasmic tail to 12 amino acids may: (i) increase mutant Env density on the cell surface thereby augmenting the number of Env molecules available for association with the assembling particles; or (ii) favor the association of the TM cytoplasmic region with Gag either by facilitating protein–protein interactions or by relieving the process of Env incorporation from the sterical constraints imposed by the size of the intact TM cytoplasmic tail.

In summary, our results demonstrate that, despite the relatively short size of the FIV TM cytoplasmic tail, small variations in the length of this region profoundly affect Env-mediated viral functions.

Materials and methods

Cell lines

293T, CrFK and MAGI-CXCR4 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS, GIBCO). MAGI-CXCR4 cells were additionally maintained in medium containing 0.2 mg/ml G418 (geneticin), 0.1 mg/ml hygromycin B and 1 μg/ml puromycin.

MAbs and antisera

Pooled sera from FIV-infected cats were kindly provided by E. Hoover. MAbs used to detect FIV Gag and CA proteins (PAK3-2C1) and FIV SU (SUFc1-30) were obtained from the NIH Research and Reference Reagent Program. To detect the FIV TM protein we used a mouse polyclonal antibody obtained in our laboratory. A peptide derived from the FIV TM ectodomain (amino acids 628–784 in the FIV Env) was expressed in *E. coli* as a fusion protein with glutathione-*S*-transferase and purified by affinity chromatography using glutathione-Sepharose 4B (GE Life Sciences). Six-week old female BALB/c mice were immunized intraperitoneally with 50 μg of the recombinant fusion protein emulsified in complete Freund's adjuvant. Two booster injections were administered intraperitoneally in incomplete Freund's adjuvant at intervals of 2 weeks. Blood was collected at the end of the immunization regimen and the presence of anti-FIV TM antibodies was assessed by Western blotting against the recombinant FIV TM-derived peptide and cell lysates of CrFK cells transfected with FIV proviral DNA.

Construction of FIV mutant proviruses

All FIV Env mutant proviruses were derived from the infectious clone FIV-14 of the Petaluma isolate (Olmsted et al., 1989). The TM cytoplasmic tail mutations were created by asymmetric PCR-based site-directed mutagenesis as we have previously described (Manrique et al., 2001, 2004) using the Elongase enzyme high-fidelity PCR mix (Invitrogen) and antisense oligonucleotides carrying stop codon mutations. Mutagenesis was performed on a *Spe*I–*Not*I restriction fragment which corresponds to nucleotides (nt) 8287–9474 of the FIV genome. Each *Spe*I–*Not*I fragment carrying the corresponding stop codon mutation was substituted for the wild-type counterpart in the parental FIV construct. The presence of the desired mutations was confirmed by DNA sequencing. To express the wild-type and mutant FIV genomes in human 293T cells, the U3 element of the 5′ long terminal repeat (LTR) was removed and the resulting proviral constructs were cloned under the control of the human immediate early cytomegalovirus (CMV) promoter using a strategy similar to that described by Poeschla and Looney (1998). Briefly, the wild-type and Env mutant molecular clones of FIV-14 in plasmid pSV-SPORT were digested with *Sal*I–*Tth*111I to remove the fragment encompassing the entire 5′LTR and the beginning of the *gag* gene (nt 1–924). Then, a *Sal*I–*Tth*111I-tailed PCR fragment extending from the R-U5 region of the 5′LTR to the *gag* gene (nt 216–924) was ligated into the *Sal*I–*Tth*111I-digested pSV-FIV-14 plasmids in order to generate the wild-type and Env mutant pSV-ΔU3FIV-14 constructs. The ΔU3FIV genomes were excised from the pSV-SPORT backbone by digestion with *Sma*I–*Not*I and cloned into the pCDNA3.1 vector

(Invitrogen), thereby placing the ΔU3FIV genomes immediately downstream of the pCDNA CMV promoter. To create a construct expressing the HIV-1 *tat* and *rev* genes under the control of the HIV-1 LTR, the *env*-minus pNL4-3 plasmid (Celma et al., 2001) was digested with *Eco*RI–*Spe*I, blunt-ended with the Klenow fragment of DNA polymerase I and religated. Thus, the *env*-minus HIV-1 genome was deleted of nt 1442 to 5742 thereby eliminating the open reading frames of the *gag* and *pol* genes.

Transfections

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

Mutant glycoprotein expression

Wild-type or mutant Env proviral DNAs were transfected into CrFK cells as described above. Transfected cells were washed twice with ice-cold phosphate-buffered saline (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), blotted onto nitrocellulose membranes and analyzed by Western blotting using the SUFc1-30 MAb to detect the FIV SU or the mouse anti-FIV TM polyclonal serum obtained in our laboratory. Horseradish peroxidase-conjugated anti-mouse immunoglobulin (GE Life Sciences) was used as secondary antibody. Western blots were developed with an enhanced chemiluminescence and chemifluorescence assay (ECL Plus Reagent, GE Life Sciences) and the resulting signal was quantitated as previously described (Manrique et al., 2003).

Cell surface biotinylation

Biotinylation of surface proteins in transfected cells was carried out essentially as we have previously described (Affranchino and González, 2006; Celma et al., 2001). Briefly, CrFK cells grown in 35-mm-diameter dishes were transfected with 4 μg of the wild-type or each of the Env mutant proviral DNAs. At 48 h post-transfection, 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; GE Life Sciences) 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 FIV-infected cats and viral proteins were separated on SDS-7.5% polyacrylamide gels and transferred to nitrocellulose membranes. Cell surface proteins were visualized by using streptavidin–horseradish peroxidase conjugate and ECL. To analyze total Env expression, a blot containing a fraction of the immunocomplexes was probed with the anti-SU MAb SUFc1-30.

Cell–cell fusion assays

To examine the ability of the mutant FIV Env glycoproteins to mediate cell-to-cell fusion, 293T cells were transiently transfected with the pCDNA-ΔU3 FIV proviral DNAs together with the HIV-1 *tat/rev* expression construct. Forty-eight hours post-transfection, the cells were dissociated and equivalent numbers of cells were added, in triplicate, at a 1:2 or 1:5 ratio to 4×10^4 MAGI-CXCR4 target cells in 24-well plates. Coculture was continued for 48 h, after which cells were stained for β-galactosidase and scored for syncytium formation as described previously (Affranchino and González, 2006). Syncytia were counted by visual microscopy at 100X 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.

Analysis of Env incorporation into virions

CrFK cells were transfected with the wild-type or Env mutant proviral DNAs and 48 h post-transfection 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., 2004). Cell- and virion-associated proteins were resolved by SDS-PAGE, blotted onto nitrocellulose membranes and analyzed by Western blotting using the PAK3-2C1 MAb to detect the FIV Gag and CA proteins, the SUFc1-30 MAb to detect the FIV SU and the mouse anti-TM polyclonal antibody to detect the FIV TM.

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