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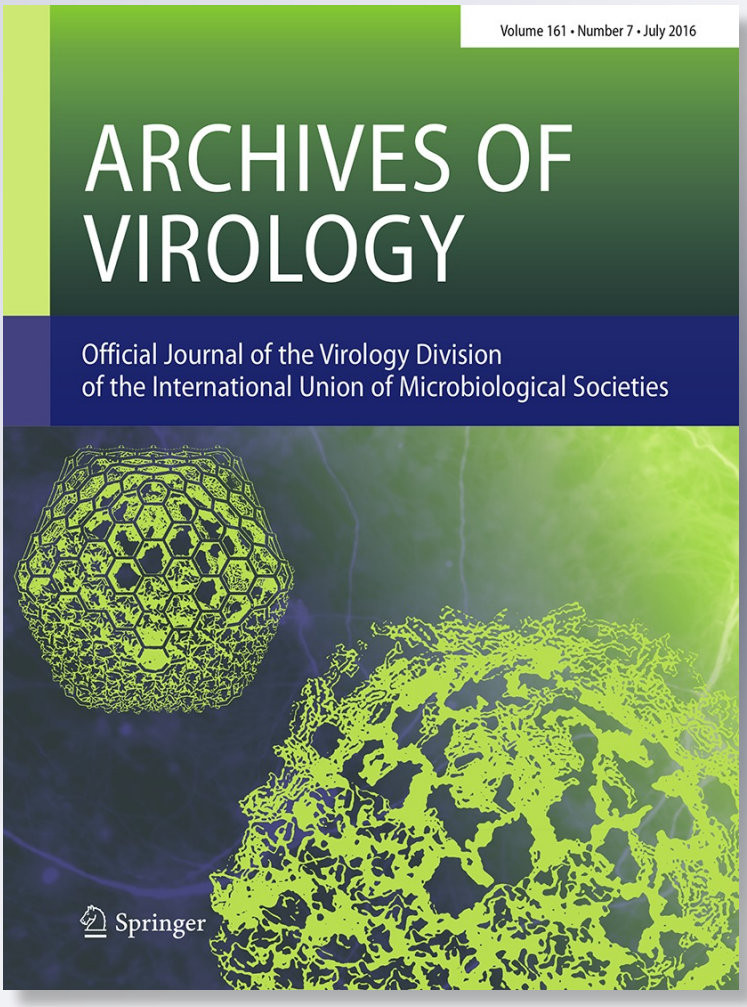

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ORIGINAL ARTICLE

Processing, fusogenicity, virion incorporation and CXCR4-binding activity of a feline immunodeficiency virus envelope glycoprotein lacking the two conserved N-glycosylation sites at the C-terminus of the V3 domain

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Abstract The process of feline immunodeficiency virus (FIV) entry into its target cells is initiated by the association of the surface (SU) subunit of the viral envelope glycoprotein (Env) with the cellular receptors CD134 and CXCR4. This event is followed by the fusion of the viral and cellular membranes, which is mediated by the transmembrane (TM) subunit of Env. We and others have previously demonstrated that the V3 domain of the SU subunit of Env is essential for CXCR4 binding. Of note, there are two contiguous and highly conserved potential N-glycosylation sites (^{418}NST^{420} and ^{422}NLT^{424}) located at the C-terminal side of the V3 domain. We therefore decided to study the relevance for Env functions of these N-glycosylation motifs and found that disruption of both of them by introducing the N418Q/N422Q double amino acid substitution drastically impairs Env processing into the SU and TM subunits. Moreover, the simultaneous mutation of these N-glycosylation sites prevents Env incorporation into virions and Env-mediated cell-to-cell fusion. Notably, a recombinant soluble version of the SU glycoprotein carrying the double amino acid replacement N418Q/N422Q at the V3 C-terminal side binds to CXCR4 with an efficiency similar to that of wild-type SU.

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Introduction

Feline immunodeficiency virus (FIV) is a lentivirus that induces in domestic cats an AIDS-like syndrome [1]. FIV, like other lentiviruses, has a single envelope glycoprotein (Env) that mediates the recognition and entry of the virus into its target cells. Translation of the FIV *env* gene yields a glycoprotein of approximately 150 kDa that is processed into a 130-kDa product by removal of an unusually long N-terminal signal peptide (Fig. 1a) [2, 3]. This polypeptide of 130 kDa, known as the Env precursor, is further processed in the trans-Golgi network into the surface (SU) and the transmembrane (TM) subunits, which remain non-covalently attached, thereby constituting the mature functional Env heterodimer (Fig. 1a) [2, 3]. Cell entry of FIV requires binding of the SU glycoprotein to the cell-surface receptor CD134 [4, 5] which initiates a series of conformational changes in Env that allow the subsequent association of the SU with the chemokine receptor CXCR4 [6, 7] and the fusion of the viral and cellular membranes mediated by the TM glycoprotein [8–10]. FIV has a broader tropism than that of human immunodeficiency virus type 1 (HIV-1), since it not only infects $CD4^+$ T lymphocytes, monocytes and macrophages but also exhibits tropism for $CD8^+$ T and B lymphocytes [11–14]. Given that monocytes and $CD8^+$ T and B lymphocytes express CXCR4 but not CD134, a selection of CD134-independent viruses may occur during the course of FIV infection in cats, which would not only expand cell tropism but would also contribute to viral pathogenesis [15, 16]. Therefore, uncovering the mechanism of the interaction between the FIV SU and CXCR4 is particularly important for understanding how viral entry proceeds in different cell types. In this regard, we and others have demonstrated that one of the six variable regions of the FIV SU, the V3

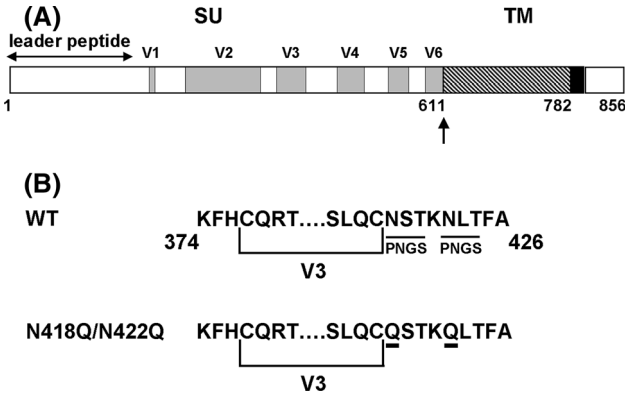

Fig. 1 Mutation of the potential N-glycosylation sites on the C-terminal side of the V3 domain. **a** Diagram of the FIV Env glycoprotein showing the leader peptide and the SU and TM subunits as well as the proteolytic cleavage site that generates these proteins (arrow). The six variable regions (V1-V6) of the SU protein are indicated by grey boxes. The components of the TM subunit, ectodomain, membrane-spanning region and cytoplasmic tail, are highlighted with hatched, black and open boxes, respectively. Amino acid numbering corresponds to the Env glycoprotein of the Petaluma FIV-14 isolate. **b** The double amino acid substitution N418Q/N422Q introduced at the C-terminus of the V3 domain of the SU glycoprotein that removes the potential N-glycosylation sites (PNGS) at positions 418-420 and 422-424 is shown below the wild-type (WT) amino acid sequence of the SU

domain, plays an essential role in the interaction between the SU and the viral coreceptor CXCR4 [17–19]. Moreover, we have shown that replacement in the FIV SU of the V3 region with the equivalent domain of a CXCR4-tropic HIV-1 results in a chimeric Env glycoprotein that not only binds to CXCR4 as efficiently as wild-type FIV Env but also promotes CXCR4-dependent membrane fusion [17].

The FIV Env glycoprotein is heavily glycosylated and contains throughout the SU subunit 16 potential N-glycosylation sites that are highly conserved among FIV isolates [20]. Studies performed on HIV-1 have demonstrated that glycans covalently attached to gp120 asparagine residues contribute to the correct processing of the Env precursor [21], allow the virus to evade the host immune response [22, 23], and facilitate the association of gp120 with the HIV-1 primary receptor CD4 [24, 25] or with coreceptors [26]. Interestingly, it has been shown that certain potent and broad neutralizing antibodies isolated from HIV-1-infected patients target conserved glycans of HIV-1 gp120 [27].

As we mentioned above, we have recently demonstrated, using a CD134-independent CXCR4-dependent FIV isolate, that deletion of the SU V3 region (Env residues 377-417; Fig. 1) eliminates the ability of the Env glycoprotein to bind to the CXCR4 receptor at the surface of the target cells without affecting Env synthesis, processing or cell surface expression [17]. Of note, there are two contiguous potential N-glycosylation sites (^{418}NST^{420} and ^{422}NLT^{424}) located immediately downstream of the C-terminus of the V3 domain (Fig. 1b). These motifs are remarkably conserved among FIV isolates, as revealed by an NCBI-BLAST search against all non-redundant protein and translated DNA sequence databases. In this report, we investigated the importance for FIV Env functions of the potential addition of N-glycans at these SU sites by studying whether mutation of these N-glycosylation motifs affects synthesis, processing and virion incorporation of FIV Env. In addition, we compared the fusogenic activity and the CXCR4-binding ability of the mutant FIV Env protein with that of the wild-type glycoprotein.

Materials and methods

Cell lines

293T, HeLa and Crandell feline kidney (CrFK) cells, obtained from the American Type Culture Collection (ATCC, USA), were grown in Dulbecco's modified Eagle's medium (GIBCO-Life Technologies) supplemented with 10 % fetal bovine serum (GIBCO-Life Technologies). MAGI-CXCR4 cells (NIH AIDS Reagent Program) were additionally maintained in medium containing 0.2 mg of geneticin disulfate (G418, Invitrogen), 0.1 mg of hygromycin B (Invitrogen) and 1 μg of puromycin (Sigma-Aldrich) per ml as described previously [8].

DNA constructs and mutagenesis

The FIV constructs were derived from the infectious molecular clone FIV-14 of the Petaluma isolate [28]. Mutation of the SU domain was performed by asymmetric PCR-based site-directed mutagenesis as described previously [8, 10] using the Elongase enzyme high fidelity mix (Invitrogen) and an antisense oligonucleotide carrying the desired mutation. The double mutation affecting the asparagine residues at Env positions 418 and 422 was introduced into a genomic DNA fragment encoding FIV Env residues 43-801 (nucleotides [nt] 6390-8669) that was amplified by PCR using specific primers. The mutated DNA fragment encoding Env residues 43-801 was digested with *Bgl*II and *Spe*I and substituted for the wild-type fragment in the FIV proviral DNA cloned in the pSV.SPORT1 vector [8], thereby generating the FIV genomic clone pSV-FIV*env*$_{N418Q/N422Q}$ lacking the potential N-glycosylation sites at positions 418-420 and 422-424 within the SU coding region.

We have recently created the plasmid pcDNA-SU$_{WT}$-HA to express a soluble form of the FIV SU containing at its C-terminus the influenza virus hemagglutinin (HA) epitope YPYDVPDYA [17]. Using pSV-FIV*env*$_{N418Q/N422Q}$ as a template, we amplified by PCR the genomic

region encompassing nt 6266-7764 (SU amino acids 1-499), and the PCR product was digested with *Bam*HI (the recognition site for which was introduced in the sense primer) and *Mfe*I (present in the FIV *env* gene). The resulting DNA fragment was used to replace the corresponding sequence in pcDNA-SU_{WT}-HA [17] digested with *Bam*HI/*Mfe*I.

For cell fusion experiments, we made use of the pcDNA-FIV*env*/*rev* plasmid, which, in addition to expressing the FIV Env and Rev proteins, includes the Rev-responsive element [8]. To generate the construct pcDNA-FIV$env_{N418Q/N422Q}$/*rev*, the *Bam*HI/*Mfe*I sequence (nt 6266-7759) of pcDNA-FIV*env*/*rev* was replaced by the corresponding fragment in which the N-glycosylation sites 418-420 and 422-424 were mutated.

All of the FIV *env*-derived constructs were confirmed by DNA sequencing.

Expression of FIV $Env_{N418Q/N422Q}$

Confluent monolayers of CrFK cells (grown in 60-mm-diameter dishes) were transfected with the wild-type and mutant $env_{N418Q/N422Q}$ proviral DNAs using Lipofectamine 2000 (Invitrogen) as reported previously [8, 10]. Forty-eight hours 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 % (v/v) Nonidet P-40, 0.1 % (w/v) SDS, 0.5 % (w/v) sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of aprotinin per ml, followed by a 5-min centrifugation at 16,000 × *g* to remove cellular debris [8]. The culture supernatants from the transfected cells were filtered through 0.45-μm syringe filters, and the virions were purified by ultracentrifugation through a 20 % sucrose cushion as described previously [8, 10]. Cell lysates, clarified supernatants and purified virions were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and analyzed by western blotting using the anti-FIV V3 monoclonal antibody (MAb) SUFc1-30 (NIH AIDS Reagent Program), a mouse polyclonal antibody directed to the TM ectodomain, prepared in our laboratory [8] or a MAb directed to the CA domain of the FIV Gag polyprotein (MAb PAK3-2C1; NIH AIDS Reagent Program). Western blots were developed with an enhanced chemiluminescence assay (Western Lightning ECL Pro; PerkinElmer), and the resulting signal was quantitated as described previously [29].

Expression of the $SU_{N418Q/N422Q}$-HA glycoprotein

The SU_{WT}-HA and $SU_{N418Q/N422Q}$-HA were expressed in 293T cells using the vaccinia T7 system as we have recently reported [17, 30]. Briefly, cells were infected for 1 h at 37 °C with the vTF7-3 recombinant vaccinia virus, which directs the synthesis of the T7 RNA polymerase [31]. After infection, cells were washed twice with DMEM and then transfected with the SU_{WT}-HA or the $SU_{N418Q/N422Q}$-HA constructs as described previously [17]. Thirty hours postinfection, culture supernatants were filtered through 0.45-μm-pore-size filters and stored at −80 °C until further use.

Cell surface biotinylation assay

Biotinylation of surface proteins in CrFK cells transfected with the wild-type or the mutant $env_{N418Q/N422Q}$ proviral DNAs was performed essentially as described previously [8, 10, 32]. Transfected cells were incubated for 30 min at 4 °C with the membrane-impermeable biotinylating reagent biotin-XX sulfosuccinimidyl ester (FluoReporter Cell-Surface Biotinylation Kit; Molecular Probes), then 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 to nitrocellulose membranes. Cell-surface proteins were detected by horseradish peroxidase (HRP)-conjugated streptavidin (USBiological Life Sciences) and ECL (Western Lightning ECL Pro; PerkinElmer)

Fusion activity

To investigate the ability of the mutant $Env_{N418Q/N422Q}$ glycoprotein to promote cell-to-cell fusion, we conducted a syncytium formation assay developed by our laboratory [8]. Briefly, 293T cells were transfected with pcDNA-env_{WT}/*rev* or pcDNA-$env_{N418Q/N422Q}$/*rev* together with an HIV-1 Tat-expressing plasmid [8]. At 48 h post-transfection, equivalent numbers of cells were added at a 1:2 ratio to 4 × 10^4 MAGI-CXCR4 indicator cells (HeLa-CXCR4-LTR-β-galactosidase [33]) in 24-well plates. After 48 h of coculture, cells were stained for β-galactosidase activity and scored for syncytium formation as reported previously [8, 10].

Cell surface binding

To investigate the association between FIV $SU_{N418Q/N422Q}$ and the cellular receptor CXCR4, we made use of a cell surface binding assay that we have recently developed, which is based on the expression of a soluble version of FIV Petaluma SU C-terminally tagged with the HA epitope [17]. The Petaluma strain is independent of CD134 and only requires CXCR4 for cell entry [5, 8]. Cell culture supernatants containing the SU_{WT}-HA or $SU_{N418Q/N422Q}$-

HA protein were incubated with HeLa cells for 1 h at 37 °C. Cells were then washed with PBS, and the cell lysates were analyzed by immunoblotting using a rat anti-HA MAb conjugated to HRP (Roche Applied Science) as reported previously [17]. Inhibition of cell surface binding of SU_{WT}-HA and $SU_{N418Q/N422Q}$-HA proteins with the CXCR4 antagonist AMD3100 (Sigma-Aldrich) was performed by first preincubating the HeLa cells for 30 min at 37 °C with DMEM containing 20 μg of AMD3100 per ml. The medium was then removed and replaced with culture supernatants containing the soluble SU-HA proteins and AMD3100 at the above-mentioned final concentration. Cells were further incubated for 1 h at 37 °C, and the presence of the SU-HA proteins in the cell lysates was assessed by western blotting as described above.

Results

Generation of a mutant FIV Env glycoprotein lacking the N-glycosylation signals located at the C-terminal side of the SU V3 domain

We have recently demonstrated, using a CD134-independent CXCR4-dependent FIV isolate, that the SU V3 region (Env residues 377-417; Fig. 1b) is essential for Env-CXCR4 interaction [17]. Of note, there are two contiguous and highly conserved potential N-glycosylation sites at the C-terminal side of the V3 domain (Fig. 1b). Therefore, to investigate the relevance for FIV Env functions of the potential addition of N-glycans at these SU sites, we first replaced the asparagines at positions 418 and 422 by glutamines so as to remove the two N-glycosylation motifs and then characterized the phenotype of the resulting mutant $Env_{N418Q/N422Q}$ protein.

Synthesis and processing of FIV $Env_{N418Q/N422Q}$

We first examined whether removal of the potential N-glycosylation sites at the C-terminus of the V3 had any effect on Env expression and processing. When lysates of CrFK cells transfected with wild-type or $env_{N418Q/N422Q}$ proviral DNAs were immunoblotted with the anti-TM antibody, we found that the mutant Env glycoprotein precursor was expressed in a wild-type manner (Fig. 2a). Indeed, the intracellular levels of the $Env_{N418Q/N422Q}$ precursor were similar to those of its wild-type counterpart (Fig. 2a). However, processing of the mutant Env glycoprotein was found to be defective, since cleavage of the $Env_{N418Q/N422Q}$ precursor yielded significantly lower levels of the TM subunit than those resulting from the processing of the wild-type Env precursor (Fig. 2a). Moreover, when the cell lysates were probed with the anti-SU MAb, higher

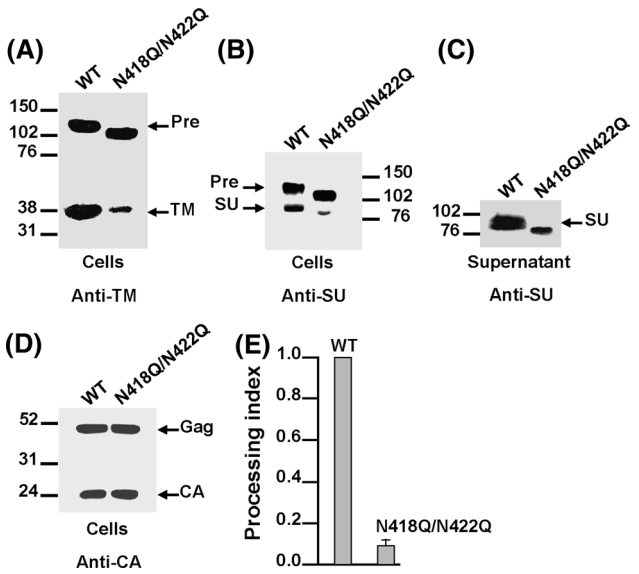

Fig. 2 Biosynthesis and processing of mutant FIV $Env_{N418Q/N422Q}$. CrFK cells were transfected with the wild-type (WT) or mutant (N418Q/N422Q) proviral DNAs, and at 48 h post-transfection, the Env proteins present in the cell lysates (cells) were detected by western blotting using **a** the anti-TM polyclonal antibody, or **b** the anti-SU MAb. **c** In parallel, the cell-free culture supernatants were probed with the anti-SU MAb. **d** The cell lysates were also analyzed for the presence of the Gag and CA proteins by immunoblotting with the anti-FIV CA MAb. **e** The amounts of Env precursor and SU proteins for wild-type and mutant N418Q/N422Q glycoproteins in the cell lysates and culture supernatants were quantitated, and the resulting values were used to calculate the 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 amount of SU detected in both cell lysates and supernatants. The mobilities of the FIV proteins Env precursor (Pre), SU, TM, Gag and CA are shown, as are the positions of the molecular weight markers (in kDa)

amounts of the wild-type SU were detected compared to those of the mutant SU glycoprotein (Fig. 2b). Similarly, the levels of SU spontaneously shed into the culture supernatant were markedly reduced in the case of $SU_{N418Q/N422Q}$ with respect to those of wild-type SU (Fig. 2c). These observations cannot be attributed to a differential ability of the anti-SU MAb (SUFc1-30) to react with the wild-type and mutant SU proteins, since this antibody recognizes the WKQRNRWEWR epitope located in the central region of the V3 domain (Env residues 394-403) [18], and it is therefore unlikely that binding of the SUFc1-30 MAb to the SU protein, resolved by SDS-PAGE prior to western blot analysis, may be influenced by the absence of N-glycans at position 418 and/or 422. Furthermore, the same relative amounts of wild-type and mutant SU proteins that were detected with the SUFc1-30 MAb (Fig. 2b) were also observed when lysates of cells expressing these proteins were probed with the SU-specific MAb clone 2-3, which recognizes the V4 region of the FIV SU (data not shown). It should be noted that both the

mutant Env precursor and $SU_{N418Q/N422Q}$ migrate faster than their wild-type counterparts (Fig. 2a, b), which indicates that the FIV SU is indeed glycosylated at one site (N418 or N422) or at both positions. The differences between the intracellular amounts of SU and TM subunits of the mutant Env glycoprotein and those of wild-type Env are not due to a higher degree of wild-type FIV protein biosynthesis relative to that of the mutant virus. Indeed, similar levels of Gag and capsid (CA) proteins were detected in both wild-type- and mutant $env_{N418Q/N422Q}$-transfected cells (Fig. 2d). Moreover, comparison of the processing indexes [8] of the wild-type and mutant-Env glycoproteins showed that the degree of $Env_{N418Q/N422Q}$ processing represents only 9 % of the wild-type value (processing index: 0.09 ± 0.04; mean ± standard deviation of three independent experiments; Fig. 2e), which confirms that the mutant Env glycoprotein exhibits a defect in SU-TM cleavage.

Incorporation of mutant $Env_{N418Q/N422Q}$ into virions

We next investigated whether preventing the addition of N-glycans at the conserved SU asparagines 418 and 422 had any effect on the process of incorporation of Env into virions, which is essential for viral infectivity [2]. CrFK cells were transfected in parallel with the wild-type and mutant proviral DNAs, and viral particles were purified from the culture supernatants as described in Materials and methods. As shown in Fig. 3a, mutation of the N-glycosylation sites at the C-terminal side of the V3 domain blocks the incorporation of the $SU_{N418Q/N422Q}$ into virions. Further analysis of virion samples with a serum directed to the TM ectodomain paralleled the results obtained with the SU-specific MAb. Indeed, virion-associated TM protein was readily detected for wild-type FIV, whereas no TM protein was found in virions purified from cells transfected with the mutant virus (Fig. 3b). As control, the particulate fractions obtained from the cells transfected with the wild-type and the $env_{N418Q/N422Q}$ genomes were probed with an anti-CA MAb, which allowed us to verify that equivalent amounts of viral particles had been compared (Fig. 3c). Moreover, analysis of the cell lysates confirmed the efficient expression of wild-type and mutant Env proteins in transfected cells (data not shown).

We then performed cell surface biotinylation assays to determine whether the relative levels of the mature wild-type and mutant Env glycoproteins at the plasma membrane of transfected CrFK cells might explain the defect in Env incorporation into virions caused by the N418Q/N422Q mutation introduced in the FIV SU. As we have previously reported for FIV and simian immunodeficiency virus, both

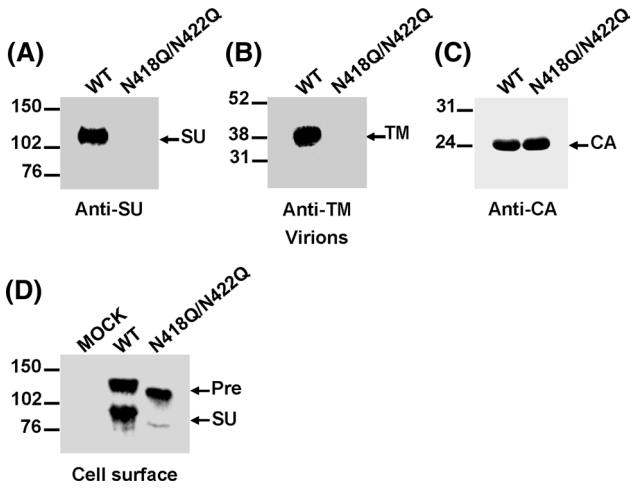

Fig. 3 Incorporation of mutant $Env_{N418Q/N422Q}$ into virions. CrFK cells were transfected with the wild-type (WT) or the mutant (N418Q/N422Q) proviral DNAs, and at 48 h post-transfection, virions were purified from the cell-free culture medium as described in Materials and methods. Virion proteins were subjected to SDS-PAGE, transferred to nitrocellulose filters and probed with **a** the anti-SU MAb, **b** the polyclonal serum directed to the TM ectodomain, or **c** the CA-specific MAb. **d** Cell surface expression of wild-type and mutant Env protein. Cells expressing these proteins were biotinylated, and Env proteins were immunoprecipitated from cell lysates as described in "Materials and methods". Biotinylated proteins on the cell surface were detected using streptavidin conjugated to HRP coupled to ECL. As control, mock-transfected cells were subjected to the same procedure

the Env precursor and the SU are readily detected by this method [8, 10, 32]. As shown in Fig. 3d, the mutant SU was barely detected at the cell surface as compared to the wild-type SU levels, which indicates that the mature (processed) $Env_{N418Q/N422Q}$ is scarcely present at the plasma membrane.

Cell-to-cell fusion mediated by the mutant FIV Env glycoprotein

To assess the effect on Env fusion activity of disrupting the potential N-glycosylation sites 418-420 and 422-424, we conducted the cell-to-cell fusion assay described in Materials and methods. When 293T cells expressing wild-type Env (together with the HIV-1 Tat protein) were added to MAGI-CXCR4 indicator cells, we detected a total of 470 ± 37 blue foci per well (mean ± standard deviation; three independent experiments; Fig. 4a). By contrast, the fusion activity of $Env_{N418Q/N422Q}$ was drastically reduced as a consequence of the mutation introduced into this glycoprotein (Fig. 4b). Indeed, the mutant Env glycoprotein promoted cell-to-cell fusion with an efficiency representing 0.75 ± 0.20 % (mean ± standard deviation of three independent experiments) of the wild-type value, which was considered 100 %.

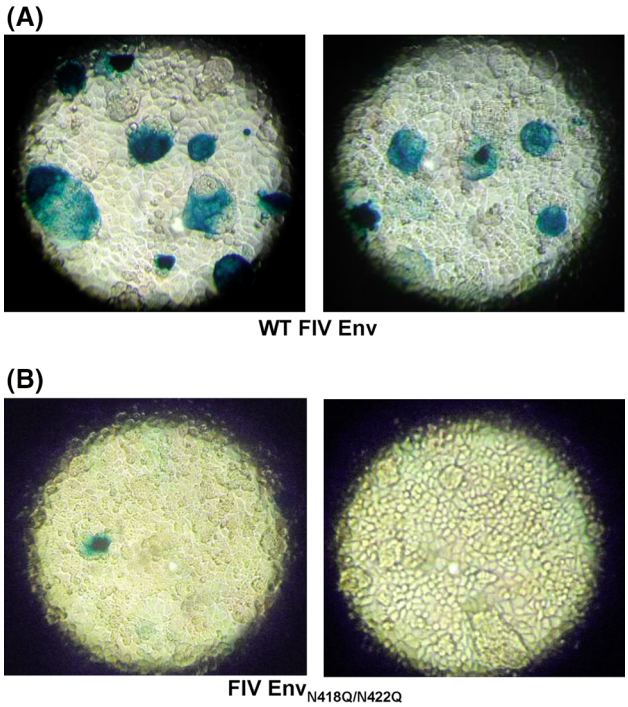

Fig. 4 Cell-to-cell fusion mediated by Env $_{N418Q/N422Q}$. 293T cells expressing the wild-type (WT) or the mutant (N418Q/N422Q) Env glycoproteins together with the HIV-1 Tat protein were dissociated, and an equivalent number of cells was added at a 1:2 ratio to 4 × 10^4 MAGI-CXCR4 cells. After 48 h of coculture, cells were stained for β-galactosidase activity and scored for syncytia formation by optical microscopy at 400× magnification. Representative images of microscopy fields are shown for the coculture of indicator cells with 293T cells expressing **a** wild-type Env, or **b** $Env_{N418Q/N422Q}$. In the latter case, blue foci were rarely detected

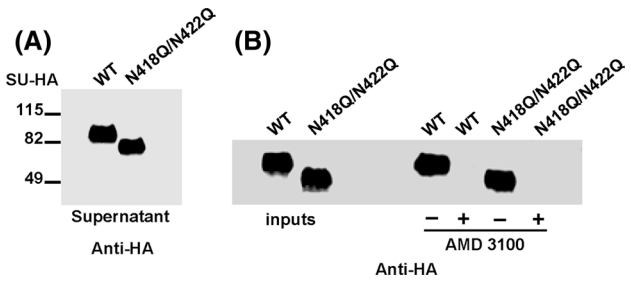

Fig. 5 Cell surface binding of SU_{WT}-HA and $SU_{N418Q/N422Q}$-HA. **a** 293T cells were transfected with the plasmid constructs directing the synthesis of soluble SU_{WT}-HA or $SU_{N418Q/N422Q}$-HA glycoproteins, and at 48 h post-transfection, the culture medium was recovered and clarified by filtration through 0.45-μm-pore-size membranes. Cell culture supernatants were subjected to SDS-PAGE, and the expression of the wild-type and mutant SU-HA proteins was detected by immunoblotting with the anti-HA MAb. **b** Aliquots of culture supernatants containing similar amounts of the SU_{WT}-HA and $SU_{N418Q/N422Q}$-HA proteins (inputs) were incubated with HeLa cells as described in Materials and methods. Cells were washed and lysed and the presence in the cell lysates of the corresponding SU-HA glycoprotein was visualized by western blotting using the anti-HA MAb. Binding of the SU-HA proteins to the surface of HeLa cells was analyzed in the absence (−) or in the presence (+) of the CXCR4 antagonist AMD3100. Inputs: as control, one-tenth of the total amount of each SU-HA protein used in the binding reactions was loaded on the gel and immunoblotted with the HA-specific antibody

Cell surface binding of the $SU_{N418Q/N422Q}$-HA glycoprotein

To study the process of FIV cell entry, we have recently developed a cell surface binding assay based on the expression of a soluble version of the FIV SU C-terminally tagged with the HA epitope [17]. The Petaluma isolate is independent of CD134 and only requires CXCR4 for cell entry [5, 8], which makes it an ideal viral strain for the study of Env-CXCR4 interaction. We have demonstrated that the method is specific for the assessment of the interaction between the FIV SU and the cellular coreceptor CXCR4 because (i) the FIV SU-HA protein only binds to cells expressing CXCR4, such as the HeLa cell line [34], and (ii) this association is inhibited by both the CXCR4 antagonist AMD3100 and the anti-CXCR4 MAb 12G5 [17]. We therefore decided to apply this methodology to examine the ability of the mutant $SU_{N418Q/N422Q}$ to interact with CXCR4. First, we generated a plasmid construct encoding a secreted version of $SU_{N418Q/N422Q}$ carrying at its C-terminus the HA epitope and then compared the expression in 293T cells of $SU_{N418Q/N422Q}$-HA with that of its wild-type counterpart. As shown in Fig. 5a, comparable levels of SU_{WT}-HA and $SU_{N418Q/N422Q}$-HA glycoproteins were detected in the culture supernatants of transfected 293T cells by western blotting using the anti-HA MAb. To investigate the CXCR4-binding capacity of the mutant SU glycoprotein, the supernatants of 293T cells containing the soluble SU_{WT}-HA or $SU_{N418Q/N422Q}$-HA proteins were incubated with HeLa cells, which are $CXCR4^+$ [34]. After the incubation period, cells were washed and lysed, and the presence of the SU-HA proteins in the cell lysates was then detected by western blotting using the anti-HA MAb. Interestingly, we found that $SU_{N418Q/N422Q}$ binds to HeLa cells as efficiently as SU_{WT} and that in both cases the association of the SU-HA to the cell surface was drastically inhibited by AMD3100 (Fig. 5b). Surface binding was inhibited to a similar extent when MAb 12G5 was used during the incubation of cells with the SU-HA proteins (data not shown). Our results therefore demonstrate that mutation of the two N-glycosylation sites present at the C-terminal side of the V3 domain does not interfere with the CXCR4-binding capacity of the FIV SU.

Discussion

In this paper, we address the role of the two contiguous N-glycosylation signals located immediately downstream of the C-terminus of the V3 domain in the biological

properties of FIV Env. Preliminary results showed that individual mutations in each of these glycosylation sites impaired Env processing and fusion activity, albeit to a lesser extent than that observed for the Env double mutant protein (data not shown). This prompted us to characterize in detail the phenotype of the FIV Env glycoproten carrying the N418Q/N422Q double mutation. Disruption of the two contiguous N-glycosylation sites at the C-terminal side of the FIV Env V3 domain causes a drastic defect in the processing of the Env precursor, resulting in significantly lower levels of cellular SU and TM proteins than those detected in cells transfected with the wild-type proviral DNA. Moreover, only trace amounts of the $SU_{N418Q/N422Q}$ protein were detected by biotinylation assays at the plasma membrane of cells expressing the mutant Env glycoprotein, which indicates that Env incorporation into virions and Env fusion activity are eliminated due to the low levels of the mature mutant SU-TM heterodimer at the cell surface. However, the recombinant soluble version of the FIV SU carrying the double mutation N418Q/N422Q was found to bind CXCR4 at the surface of HeLa cells as efficiently as wild-type SU. Taken together, these results indicate that the loss of essential functions by FIV $Env_{N418Q/N422Q}$ was not caused by a direct effect of the mutation on the structural features of SU or V3 or their biological properties. Instead, the mutation appears to act in the context of the Env precursor rendering the SU-TM cleavage site less accessible to the furin-like proteases that mediate Env cleavage in the trans-Golgi compartment, which in turn results in insufficient cellular levels of mature functional Env protein.

In conclusion, our results demonstrate that the remarkable conservation among FIV isolates of the N-glycosylation sites at Env positions 418 and 422 preserves efficient Env precursor processing and thus Env incorporation into virions and fusion activity, which are essential for virus infectivity, tropism and pathogenesis.

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Compliance with ethical standards

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Conflict of interest Both authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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