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Original Research Paper

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THE REMOVAL OF A SINGLE N-LINKED GLYCOSYLATION IN FELINE IMMUNODEFICIENCY VIRUS (FIV) V1/V2 ANALOGUE LOOP OF ENV GREATLY AFFECTS VIRAL INFECTIVITY

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ABSTRACT Viral envelope (Env) of Feline Immunodeficiency Virus (FIV) is heavily glycosylated, such that carbohydrate comprises a great proportion of its molecular weight. It is widely believed that Potential N-Linked Glycosylation Sites (PNGSs) play an important role in viral infectivity as well as protection from neutralization by shielding highly conserved immuno-dominant epitopes on Env. Here, the sequence of Env for a primary strain, designated SX95, was analysed. Interestingly, Env sequence shows a missing PNGS at position 298 at the crown of V1/V2 analogue loop; a mutation that has not previously encountered, and thought to be rare, if not unique. Remarkably, when this PNGS was reinstated, by the construction of a pseudotyped virus made of SX95's Env and a backbone of HIV with luciferase reporter gene, viral infectivity surged by 100 times compared to the wild-type variant. Further, the deletion of this PNGS at position 298 from a primary (SX08) strain rendered the virus less infectious by about the same factor. Such data suggest that this strategically placed PNGS at position 298 on Env must be a key component in boosting viral infectivity, perhaps by providing better interaction to the host's primary receptor or co-receptor. The role of this mutation on protecting the virus from neutralization is to be studied further.

KEYWORDS : FIV, HIV, Glycosylation, Env

INTRODUCTION

Although an effective vaccine was developed relatively shortly after the initial isolation of COVID-19, vaccination against persistent lentiviral infections represents a more significant challenge. Although the Human Immunodeficiency Virus (HIV) has been recognized for decades, HIV infection cannot yet be controlled by effective vaccines. A major obstacle to the development of an effective HIV vaccine is the resistance of primary viral isolates to antibody-mediated neutralization(Wyatt et al., 1998). Feline Immunodeficiency Virus (FIV) is studied as an animal model for HIV infection, being the only virus that induces immunodeficiency in its natural host, i.e. the domestic cat.

HIV Env is heavily glycosylated, where glycan residues attach to asparagine (N) residues within amino acid sequence of Env. such that carbohydrate comprises a great proportion of its molecular weight, estimated as more than 50% in HIV-1(Geyer et al., 1988; Leonard et al., 1990). It has been demonstrated that numerous potential N-linked glycosylation sites (PNGSs) are highly conserved amongst viral isolates, indicating that specific glycans may be vital for the integrity and conformation of Env, while simultaneously masking key targets for neutralizing antibodies (NAbs)(Cheng-Mayer et al., 1999). A proposed mechanism of HIV escape from NAbs is that the repositioning of glycosylation sites across the Env sequence limits recognition by NAbs but does not affect the ability of Env to interact with the primary and co-receptors(Wei et al., 2003).

Env is the target of neutralizing antibodies; however, it is poorly immunogenic(Kolchinsky et al., 2001) and evolves rapidly in response to immune pressure and selection, such that neutralization determinants continuously diminish with time post infection. Furthermore, the masking of variable loops on conserved epitopes, such as the primary and co-receptor binding sites, the occlusion of protein surfaces through trimer formation, the glycan shield formed by extensive N-linked glycosylation, and the use of a two-receptor entry mechanism all contribute to the poor immunogenicity of Env(Chackerian et al., 1997; Koch et al., 2003). In particular, the co-receptor binding site is exposed only transiently following the conformational change that is triggered by the engagement of the primary receptor(Willett et al., 2009), minimizing the time during which the binding site may be recognized by the host's immune system.

In this study, we approved that the elimination of an asparagine-linked glycosylation site at residue 298 Env, which is sited at the crown of the V1/V2 loop analogue of FIV Env, has significantly reduced viral infectivity using a susceptible cell line, CLL-CD134. The lack of PNGS at this site was first encountered in a primary isolate, designated SX95, as a result of an N298S mutation.

METHODS

Cloning and pseudotype virus production. DNA was extracted from PBMCs by using a DNA Whole Blood extraction kit (Qiagen). The FIV env gene was amplified by PCR, using forward and reverse primers containing recognition sites for the restriction enzymes SalI and NotI, and then cloned into a low-copy-number eukaryotic expression vector, VR1012 (VICAL Inc.). To prepare HIV(FIV) pseudotypes, 3.3 x 10^5 HEK-293T cells were transfected with 5 mg VR1012 expressing the FIV env clone and 5 mg pNL-Luc-E-R+(Connor et al., 1995) (an Env-deleted HIV provirus that incorporates a luciferase reporter gene) by using SuperFect reagent (Qiagen), following the manufacturer's instructions. Culture supernatants were harvested at 48–72 h post-transfection, filtered at 0.45 mm and frozen at -80 °C until required.

DNA sequencing. The FIV env genes were sequenced by using a BigDye Terminator v1.1 kit (Applied Biosystems). The reaction mixture consisted of 100 ng purified DNA per 1, 3.2 pmol each primer, 4 µl sequencing buffer and 2 l sequencing enzyme in 20 ml reactions. Cycling conditions were one cycle at 96 °C for 1 min, followed by 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Sequencing was performed by using an ABI3700 automated capillary array sequencer (Applied Biosystems). Raw chromatographic data were analysed by using 'Contig Express' sequence analysis software within the Vector NTI suite of programs (Invitrogen).

Viral infectivity. Fifty µl of luciferase HIV(FIV) pseudotypes were co-incubated in triplicate wells of 96-well flat-bottom CulturPlate-96 assay plates (Perkin Elmer) with 50 µl CLL-CD134 cells at the count of 5 x 10^5 cells per µl(Willett et al., 2006) for 1 h at 37 °C. At 72 h post-infection, 100 µl Steadylite HTS (Perkin Elmer) luciferase substrate was added to each well and luciferase activity was quantified by single photon counting on a MicroBeta luminometer (Perkin Elmer).

Site-directed mutagenesis. Mutations were introduced into the FIV env gene by using a QuikChange II Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. Briefly, each 50 µl reaction contained 50 ng DNA

(VR1012 plasmid carrying the FIV env gene), 125 ng each primer, 1x Pfu reaction buffer, 1 1 dNTP mix and 2.5 U PfuUltra high-fidelity DNA polymerase. Cycling temperatures were as follows: initial denaturation at 95 °C for 30 s, followed by 12–18 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min and extension at 68 °C for 7 min with no final extension hold. The product was then treated with DpnI endonuclease in order to digest the methylated parental DNA template. DpnI treated DNA was then transformed into XL1-Blue Supercompetent cells and the env gene was sequenced to confirm the presence of the desired mutation. Oligonucleotide primers for mutagenesis are ggaaaagtaagtatatcattatgtctaacagg and cctgttagacataatgatatacttacttttcc as antisense and sense, respectively.

RESULT

A routine inspection of viral infectivity on a susceptible cell line, CLL-CD134, for a panel of recently isolated primary FIV variants with Env pseudotyped using a backbone of HIV with luciferase reporter gene (luciferase HIV[FIV] pseudotypes), led to a remarkable observation. One variant, designated SX95, consistently showed very weak luciferase activity compared to the rest of the panel, i.e. it produced very poor viral yield and thus suboptimal infectivity (Figure 1). Such a remark made the study of Env for this variant inevitable. Hence, amino acid sequence of Env for SX95 was investigated and compared to Env sequence for primary variants isolated in our lab, as well as to those reported in literature. The goal was to identify any extraordinary changes in the sequence within "hot-spot" regions, or to check for PNGSs that might be added or taken off throughout variable loops of Env.

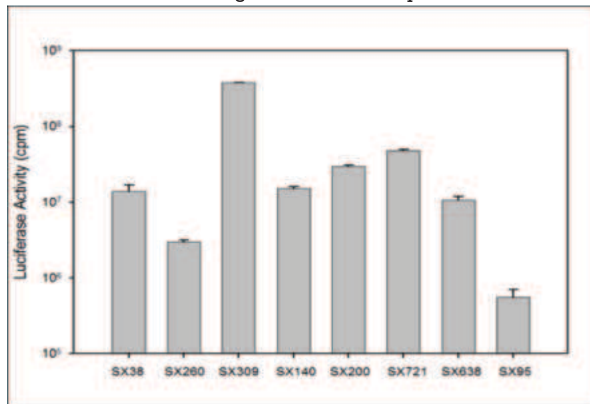

Figure 1. Comparison of the luciferase activity of the pseudotypes described in this article. Pseudotypes were incubated with CLL-CD134 cells in triplicate. Each column represents the mean (n=3) +/- SEM.

Amino acid sequence analysis revealed that SX95 Env contained fewer PNGSs than most other variants. Two missing PNGSs, in particular, caught more attention due to their positions on Env and thus were further investigated (Figure 2). Both deleted PNGSs belonged to the region analogous to the variable domains V1/V2 of HIV and SIV as shown in Figure 3.

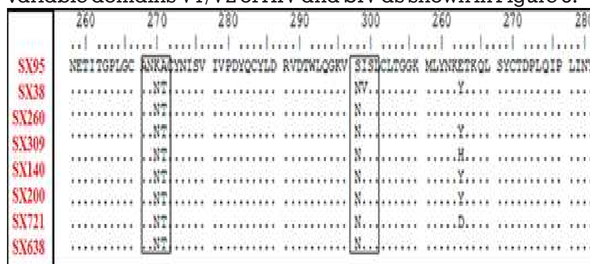

Figure 2. Amino acid sequence of V1/V2 homologue for a panel of FIV variants. The sequence of SX95 shows the loss of two PNGSs (within boxes) at sites 269 and 298.

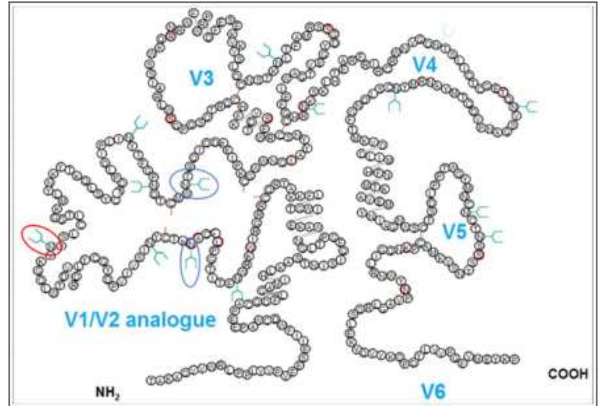

Figure 3. A schematic drawing of FIV Env showing the locations of PNGSs within variable loops in a model FIV variant, SX08. PNGSs 269 and 342 (circled in blue) are missing in FL4, the vaccine isolate, while the unique loss of PNGS at 298 in SX95 is circled in red.

The first of two missing PNGSs was investigated next. PNGS at 269 site was lost as a result of a T271A mutation, a replicate to one of two lost PNGSs (269 and 342) in FL4 variant, from which the only FIV vaccine, Fel-O-Vax FIV, was derived. Restoring this PNGS did not improve viral infectivity, however (data not shown). The second missing PNGS is located on the crown of V1/V2 domain, which occurred as a result of an N298S mutation. This seemed a unique mutation as it was not naturally encountered before neither in our database nor in any published FIV Env sequence to date.

Hence, lost PNGS at residue 298 for SX95 Env (SX95-S298N) was repaired, and viral infectivity was tested again on CLL-CD134 cells. Incredibly, this mutation restored viral infectivity by almost 100x in comparison to the wild-type variant (SX95-WT) (figure 4). This data strongly suggested that reduction in infectivity for SX95-WT, as indicated by luciferase activity, is merely attributed to the elimination of the glycan residue at 298. To verify this further, the same mutation that targets the elimination of PNGS at 298 site for the model FIV variant, SX08 was replicated. Viral activity of the mutant SX08 (SX08-N298S) was then verified. Knocking out this PNGS rendered the variant way too weaker and luciferase activity was sharply reduced (Figure 4).

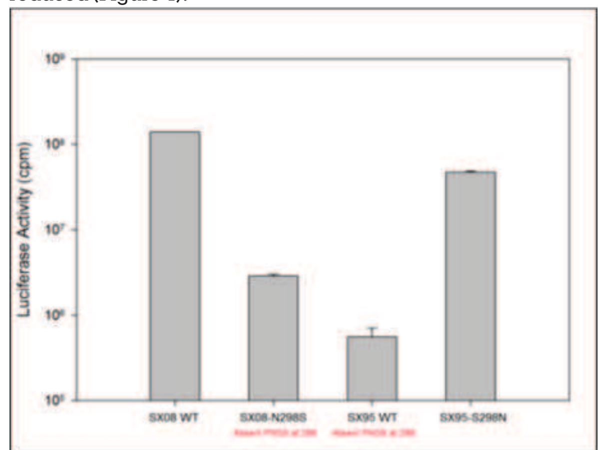

Figure 4. Comparison of luciferase activity of pseudotypes SX08 and SX95 when PNGS at 298 site is present or absent. Each column represents the mean (n=3) +/- SEM.

DISCUSSION

In this paper, we proved that the elimination of a single Potential N-linked Glycosylation Site (PNGS) at the crown of the V1/V2 homologue of FIV Env at position 298 led to a substantial reduction in viral infectivity. This PNGS at 298 site appears to be

highly conserved amongst published sequences and is believed to be vital for viral infectivity and survival.

It is established that viral evolution in response to immune pressure involves the addition and repositioning of PNGS locations within regions sometimes termed as "hot spots" (Zhang, 2004). The V1/V2 domain of HIV has been the subject of extensive investigations in this regard, since it varies greatly in both amino acid sequence and length amongst viral strains(Fox et al., 1997; Wang et al., 1995), as well as playing a role as an immunological shield covering the bridging sheet between the inner and outer domains of Env(Kwong et al., 1998), thereby protecting conserved regions of Env such as the primary and co-receptor binding sites.

A review of the literature shows that several studies have involved the removal, repositioning, and addition of potential glycosylation sites, or even partial or full deletion of variable loops, in HIV and SIV. For example, some studies have shown that glycosylation often results in the masking of certain neutralisation epitopes (Chackerian et al., 1997; Cheng-Mayer et al., 1999; Lue et al., 2002).

Moreover, it has been reported that partial deletion of V1/V2 of HIV-1 Env renders the virus less virulent, probably by redirecting immune responses toward the unshielded immunodominant epitopes and so broadening neutralising responses to Env that cannot be afforded by the full removal of the variable domains(Gzyl et al., 2004). Single and multiple alterations in numbers and locations of PNGSs showed variable success. While no significant improvement of immunogenicity of Env was obtained by mutants compared to the wild-type in many studies(Lue et al., 2002; Quiñones-Kochs et al., 2002), other studies showed some success that certain PNGSs played a role in viral susceptibility to neutralisation(Li et al., 2008; McCaffrey et al., 2004). However, no published data has yet proved that a single PNGS may produce such a major effect on viral infectivity for either FIV or HIV viruses.

It has been highlighted that critical positions of PNGSs within Env plays vital role in shielding the immunodominant determinants and so controls viral susceptibility to neutralization, and that removal or shifting the locations of these key PNGSs does not only expose localized epitopes but rather conferred structural changes on the entire Env that increase its recognition by neutralizing antibodies(Kraft et al., 2007).

This *de novo* mutation occurred in an FIV variant (SX95) that was isolated from a cat naturally infected with FIV with a serum that contained strongly and broadly neutralizing antibodies. Thus, the elimination of this PNGS at 298 asparagine residue perhaps induced a structural modification on Env that exposed a key determinant in other domains of Env (perhaps the highly conserved sequence of co-receptor) to NAbs or reduced the masking by V1/V2 domain on a principal neutralization determinant making the cat's immune system generate such a strongly, broadly neutralizing response. The effect of this PNGS on virus neutralization must be investigated further.

Moreover, the study of the map of PNGSs throughout Env amongst published amino acid sequences will benefit in identifying all highly conserved sites, particularly those located in variable loops of Env. Perhaps the elimination of these PNGSs individually or following certain patterns may decode at the extent of effect on Env immunogenicity and viral infectivity the glycan confers. The combination of elimination of one or more PNGSs may resemble the ideal immunogen that generates broadly neutralising response, and could pave the way towards generating an efficacious vaccine.

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