Mutational analysis of a principal neutralization domain of visna/maedi virus envelope glycoprotein

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We have shown previously that a type-specific neutralization domain is located within a 39 aa sequence in the fourth variable domain of gp135 in visna/maedi virus. We now show that neutralizing antibodies detected early in infection are directed to this epitope, suggesting an immunodominant nature of this domain. Ten antigenic variants were previously analysed for mutations in this region, and all but one were found to be mutated. To assess the importance of these mutations in replication and neutralization, we reconstructed several of the mutations in an infectious molecular clone and tested the resulting viruses for neutralization phenotype and replication. Mutation of a conserved cysteine was shown to alter the neutralization epitope, whilst the replication kinetics in macrophages were unchanged. Mutations modulating potential glycosylation sites were found in seven of the ten antigenic variants. A frequently occurring mutation, removing a potential glycosylation site, had no effect on its own on the neutralization phenotype of the virus. However, adding an extra potential glycosylation site in the region resulted in antigenic escape. The results indicate that the conserved cysteine plays a role in the structure of the epitope and that glycosylation may shield the principal neutralization site.

INTRODUCTION

Visna/maedi virus (VMV) is a macrophage-tropic lentivirus causing slowly progressive diseases that mainly affect the lungs and the central nervous system of sheep (Gorrell et al., 1992; Sigurdsson, 1954). VMV establishes a lifelong infection and persists in the host despite a strong immune response. In an VMV infection, a type-specific neutralizing antibody response appears 1–6 months after infection, whilst more broadly reacting antibodies appear up to 4 years later (Andresdottir et al., 2002). A similar antibody response has been reported for human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) (Moog et al., 1997; Nara et al., 1990; Rudensey et al., 1998).

The envelope glycoproteins of the lentiviruses are highly variable proteins with a conserved conformation. The protein structure is maintained, with conserved cysteine residues and glycosylation (Li et al., 1993; Tschachler et al., 1990), and there is some structural similarity between the envelope glycoproteins of all lentiviruses (Gallaher et al., 1995; Hotzel & Cheevers, 2001). There are more than 20 potential N-linked glycosylation sites in the outer glycoprotein of VMV, as well as in those of other lentiviruses. It has been suggested that the role of this extensive glycosylation is to shield the virus from neutralizing antibodies (Reitter et al., 1998; Wei et al., 2003). This was first suggested by Huso et al. (1988), who showed that whilst treatment of caprine arthritis encephalitis virus (CAEV) with neuraminidase did not reduce infectivity, it enhanced the kinetics of neutralization of the virus by goat antibodies. Indeed, the lentiviruses seem to have evolved many mechanisms of immune evasion (Frost et al., 2005; Kwong et al., 2002; Wei et al., 2003; Wyatt & Sodroski, 1998). One way for the lentiviruses to escape the immune response may be by continuous change of epitopes through mutation, as first proposed for VMV by Gudnadottir et al. (1974) and further confirmed and extended by Narayan et al. (1977, 1978, 1981). A wealth of genetic, immunological and structural studies of HIV-1 envelope glycoproteins have revealed remarkable diversity and conformational flexibility of these molecules that may result in neutralization escape, either by mutation of the neutralization epitopes or indirectly by conformational masking of epitopes or shielding by glycosylation (Huang et al., 2005; Kwong et al., 2002; Wei et al., 2003; Wyatt & Sodroski, 1998; Wyatt et al., 1998).

The initial type-specific neutralizing antibodies detected in the sera of HIV-1-infected humans are mostly directed to the V3 regions on gp120, hence the term ‘principal neutralization domain’ (PND) (Javaherian et al., 1989). The V3 region plays a central role in determining coreceptor usage and viral tropism (reviewed by Hartley...
et al., 2005). Early antibodies are also directed to the V1/V2 variable loops, whereas later, more broadly reacting antibodies are probably directed mostly to receptor-binding site surfaces (Wyatt & Sodroski, 1998). In SIV, the V1 and V4 variable regions seem to contain the principal neutralizing determinants (Kinsey et al., 1996; Rudensky et al., 1998). We have mapped mutations altering an early type-specific neutralization response of the VMV strain KV1772 to the fourth variable region of VMV (Skraban et al., 1999).

The Icelandic VMV strains K1514 and K1772 induce a much stronger neutralizing antibody response than other strains of VMV or CAEV (Cheevers et al., 1991, 1993; Narayan et al., 1984). These virus strains are therefore well suited for determining the effect of neutralizing antibodies in a VMV infection. In a previous study from this laboratory, 20 sheep were inoculated with VMV strain K1514, and virus was isolated from blood and cerebrospinal fluid over a period of 7.5 years. All of the sheep mounted a strong, strain-specific neutralizing antibody response 2–5 months after infection, but more broadly reacting neutralizing antibodies appeared up to 4 years later. Most virus strains that were isolated from the sheep up to 7 years after infection were neutralized by the early, type-specific antisera. However, 10 of 61 virus isolates from blood were antigenic variants (Lutley et al., 1983). The neutralization domain was found to be mutated in nine of the ten antigenic variants (Andresdottir et al., 2002). In this study, we investigated the importance of a conserved cysteine and potential glycosylation changes occurring in the antigenic variants in replication kinetics and neutralization phenotype.

METHODS

Virus and cells. The molecularly cloned virus KV1772 (formerly KV1772kv7267) has been described previously (Andresson et al., 1993; Skraban et al., 1999). Sheep choroid plexus (SCP) cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 2 mM glutamine, 100 IU penicillin ml⁻¹, 100 IU streptomycin ml⁻¹ and either 10% (growth medium) or 1% (maintenance medium) lamb serum. Macrophage cultures were established from heparinized blood by sedimentation on Histopaque-1077 (Sigma) as described previously (Skraban et al., 1999).

Reverse transcriptase (RT) assay. Virus particles from 0.5 ml cell-free supernatant from infected cells were pelleted at 14,000 r.p.m. at 4°C for 1 h in a microcentrifuge. RT activity was determined by measuring the incorporation of [³H]TTP in the extension of oligo(dT), using poly(rA) as a template as described previously (Andresdottir et al., 1998).

Neutralization assay. Neutralization assays were carried out with monolayers of SCP cells in 96-well tissue-culture plates as described previously (Skraban et al., 1999). Briefly, 100 TCID₅₀ virus in 0.1 ml DMEM with 2% normal lamb serum was mixed with 0.1 ml of a twofold dilution of serum sample from infected sheep. The mixtures were incubated at room temperature for 20 h and each dilution was inoculated in quadruplicate onto monolayers of SCP cells. Control cultures were inoculated with virus in the absence of antisera. Cytopathic effect was monitored after 7, 14, 21 and 28 days. Neutralization titre was calculated as the reciprocal of the serum dilution that caused complete neutralization in 50% of inoculated cultures.

Construction of mutant viruses. The molecular clone KV1772 is contained in two plasmids, as has been described previously (Skraban et al., 1999). Mutations altering the potential glycosylation sites and introducing the C-Y change in the neutralization domain were generated by PCR-mediated site-directed mutagenesis. The presence of the mutations was confirmed by DNA sequencing.

DNA transfections were performed with monolayers of ovine fetal synovial (FOS) cells. Lipofectamine 2000 was used as specified by the manufacturer (Invitrogen). Supernatants from transfected cells were tested periodically for RT activity.

RESULTS

The V4 neutralization domain is immunodominant

The envelope gene of VMV is divided into conserved and variable regions. The variable regions of the env gene of the small ruminant lentiviruses are shown in Fig. 1, as identified in CAEV by Valas et al. (2000). The positions of V4 and V5 are in good agreement with results that we and others have obtained from studies of variation in VMV, whereas the positions of V1–V3 differ somewhat between studies (Andresdottir et al., 1998; Gjerset et al., 2007; Mwaengo et al., 1997; Sargan et al., 1991). We

![Fig. 1. Schematic representation of the surface unit of the envelope gene, showing variable regions as determined by Valas et al. (2000) (black boxes labelled V1–V5) and potential sites for N-linked oligosaccharides (denoted by forks). The amino acid sequence of the principal neutralization domain (PND) is shown. The two conserved cysteines that are proposed to form a cysteine loop are underlined.](image-url)
previously mapped a type-specific neutralization epitope to a region of 39 aa in the fourth variable domain of gp135 (Skraban et al., 1999). To determine whether this region defined an immunodominant epitope, a number of early sera from sheep that had been inoculated with either the virus strain K1514 or the homologous, molecularly cloned virus KV1772 were tested for neutralization of the ‘wild-type’ KV1772 or strain VR1, which has multiple mutations within the 39 aa epitope region in a KV1772 background. We found that whilst KV1772 was neutralized by all sera at a high titre, VR1 escaped all early sera and was only partly neutralized by a serum taken 30 months after inoculation. However, VR1 was neutralized readily by autologous antiserum (Table 1). It thus appears that early sera are directed against this epitope.

**Mutation of a conserved cysteine resulted in antigenic escape**

A number of antigenic variants were previously shown to contain multiple mutations in the epitope region (Fig. 2) (Andredottir et al., 2002). The region contains two conserved cysteines previously suggested to form a cysteine loop (Fig. 1) (Skraban et al., 1999). Two of the antigenic variants had alterations of one of the conserved cysteines. In one strain, 1518-108, there was a deletion of the cysteine, and in strain 1521-165, a C–Y change had occurred. In both strains, the cysteine change was accompanied by other mutations. To assess the importance of the cysteine in replication proficiency and in the ability of the virus to escape neutralization, a mutation where the cysteine was changed to tyrosine was generated in the molecular clone KV1772. This mutant was called BS4 (Fig. 2). Replication of the mutant virus was examined in blood-derived macrophages and compared with that of parental KV1772. The mutation did not have any detectable effect on the replication proficiency of the virus (Fig. 3). Next, the neutralization sensitivity of the virus was tested. We use SCP cells routinely for testing neutralization and we have shown that the neutralization specificity is unchanged whether we use SCP cells or macrophages as host cells (Skraban et al., 1999). As shown in Table 2, the C–Y mutation rendered the virus completely resistant to neutralization by the type-specific antiserum. We have previously shown that the neutralization epitope is conformational and it is likely that, by changing the cysteine, a disulfide bridge is disrupted (Skraban et al., 1999).

**Mutation of a conserved potential glycosylation site had no effect on the neutralization phenotype, whereas addition of a potential glycosylation site conferred neutralization resistance**

The glycosylation site at aa 568 in VMV gp135 is highly conserved in all VMV and CAEV strains that have been sequenced. This site was mutated in five of the antigenic variants through at least four independent mutations (Fig. 2). The mutation from isolate 1548-123, S–R, in the potential glycosylation site NES was generated in the backbone of KV1772. This mutant was called BS5. As shown in Table 2, there was no change in neutralization phenotype. It appears likely that the mutation acts in concert with other mutation(s) outside the region that was sequenced in the antigenic variants.

**Table 1. Neutralization of KV1772 (autologous) and VR1 (heterologous) by early sera**

Neutralization of the VMV molecular clones KV1772 and VR1 by early sera from sheep infected with KV1772 (sheep nos 1968 and 1954) and the isotypic VMV strain K1514 (sheep nos 1520, 1521 and 1548). Serum from a sheep infected with the heterologous strain VR1 is also shown. Neutralization titre was determined as described in Methods and is shown as the reciprocal of the serum dilution that caused complete neutralization in 50% of inoculated cultures. Data represent one of three independent neutralization experiments.

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<th>Serum (sheep no.)</th>
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<td>1998 (inoculated with VR1)</td>
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**Fig. 2.** Amino acid sequences in the neutralization domain of the ten antigenic variants (top) and the mutations introduced into molecular clones (bottom). Potential N-linked glycosylation sites are shown by a bar over the sequence.
Another frequently occurring mutation in the antigenic variants created an additional potential glycosylation site, sometimes accompanying a mutation abolishing the conserved glycosylation site. This resulted in either two glycosylation sites 7 aa apart or a shift of the glycosylation site by 7 aa (Fig. 2). The additional potential glycosylation site was generated by introducing a K–N substitution in the sequence KCS, generating an NCS glycosylation site. This was done on the backbone of both KV1772 (resulting in two glycosylation sites) and the mutant BS5 (resulting in a shift of glycosylation site). These strains were called BS16 and BS17, respectively (Fig. 2). Table 2 shows the neutralization phenotypes of the three glycosylation mutants BS5, BS16 and BS17. The strain with the conserved glycosylation site mutated was neutralized fully by type-specific serum, and so was BS17, with the glycosylation site shifted. However, the mutant virus with two glycosylation sites (BS16) escaped neutralization. The replication proficiency of BS16 was tested and compared with that of the parental strain KV1772 and BS17. Sheep blood-derived macrophages were inoculated with the virus strains and replication kinetics were examined. As shown in Fig. 4, the mutant viruses replicated with similar kinetics to the parental KV1772 strain.

**DISCUSSION**

We have shown that neutralizing antibodies detected early in infection in all five sheep tested were directed to an epitope in the fourth variable domain (V4) of VMV gp135, suggesting the immunodominant nature of this epitope (Skraban et al., 1999). The epitope region contains two conserved cysteines and a conserved potential glycosylation site. We previously reported frequent mutations in one of the conserved cysteines and in the glycosylation site in antigenic variants (Andresdottir et al., 2002). By introducing these mutations into the backbone of the molecular clone KV1772, we have shown that the cysteine is important for the structure of the epitope. We chose to change the cysteine to tyrosine, as this was a mutation found in one of the antigenic variants. We have previously suggested that this cysteine participates in a disulfide bridge, forming a loop that may have a function analogous to that of V3 in HIV-1. We cannot discern whether the neutralization escape was caused by a disrupted disulfide bridge or by changing the amino acid per se. However, two independent escape mutations of this cysteine may indicate that it is important in the conformation of the epitope.

It has been shown by a number of studies with both HIV and SIV that neutralization resistance can be conferred by glycosylation masking the epitopes (Back et al., 1994; Cheng-Mayer et al., 1999; Reitter et al., 1998; Wei et al.,...
2003). In seven of the ten antigenic variants, we found mutations modulating glycosylation sites of the neutralization domain. The region contains one potential glycosylation site that is well conserved in all sequenced strains of VMV and CAEV (Valas et al., 1997). Yet, this site was mutated in five of the ten antigenic variants. However, disruption of this glycosylation site in a KV1772 background had no effect on neutralization phenotype. This mutation must therefore act in concert with other mutation(s), either within the region or at a distant site. Shifting the glycosylation site by 7 aa, a mutation that occurred in three of the antigenic variants, also had no effect by itself. Two antigenic variants had the two potential glycosylation sites 7 aa apart. This mutant virus was fully replication-proficient in macrophages, but escaped neutralization by the KV1772 strain-specific antiserum. Although it has not been shown that both glycosylation sites are used, by analogy with HIV-1, it seems likely that added glycosylation masks the epitope. The surface glycoprotein of KV1772 has 24 potential glycosylation sites distributed non-randomly, similar to HIV (Fig. 1). The gp120 core of HIV is divided into an inner domain and an outer domain with a short bridging sheet. The inner domain binds antibodies that are non-neutralizing and is referred to as the non-neutralizing face; part of the outer domain is heavily glycosylated and is probably recognized by the host immune system as ‘self’ and is not immunogenic, whilst the surface that interacts with neutralizing antibodies involves parts of both domains and the bridging sheet and includes the V1/V2 and V3 variable loops (Wyatt & Sodroski, 1998). As the secondary/tertiary structure or the receptor-binding site(s) of VMV gp135 are not known, we can only speculate about the relationship of the neutralization domain to the receptor-binding site. Hotzel & Cheevers (2003, 2005) have provided evidence for similarities in the structures of VMV gp135 and HIV-1 gp120. The heavily glycosylated region on gp135 probably forms the outer region, as suggested by Hotzel & Cheevers (2005), and we suggest that the neutralization region constitutes a loop that shields a receptor-binding site analogous to V3 in HIV.

In summary, our studies provide evidence for the importance of the conformational structure of the PND as antigen for neutralizing antibodies. Furthermore, our studies support a role of glycosylation and a changing ‘glycan shield’ in VMV antibody escape. It thus appears that all lentiviruses may use similar strategies for immune evasion and that the Icelandic VMV strains that persist in the host despite high titres of neutralizing antibodies may serve as a useful model for the study of lentiviral persistence in the face of strong immune responses.

ACKNOWLEDGEMENTS

This study was supported by the Icelandic Research Fund and the University of Iceland Research Fund. We thank Svava Hogadottir for expert technical help.

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