Removal of the cleavage site of recombinant feline immunodeficiency virus envelope protein facilitates incorporation of the surface glycoprotein in immune-stimulating complexes

Guus F. Rimmelzwaan,1 Kees H. J. Siebelink,2 Robin C. Huisman,1 Bernard Moss,3 Michael J. Francis4 and Albert D. M. E. Osterhaus1*

1 Department of Virology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands, 2 Laboratory of Immunobiology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands, 3 Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892, U.S.A. and 4 Pitman-Moore Ltd., Breakspear Road South, Harefield, Uxbridge, Middlesex UB9 6LS, U.K.

Recombinant vaccinia viruses were constructed that expressed the complete env gene of feline immunodeficiency virus with or without the nucleotide sequence encoding the cleavage site between the surface (SU) protein and the transmembrane (TM) protein. The removal of this cleavage site resulted in the expression of a 150K protein that was processed into a 130K protein and was not cleaved into the SU and the TM proteins. Removal of the cleavage site also facilitated incorporation of the SU protein in immune-stimulating complexes (iscoms). Antibody responses to both an SU and a TM peptide representing two immunodominant B cell epitopes were measured. These were higher in cats immunized with iscoms prepared from the cleavage site-deleted envelope protein than in cats immunized with iscoms prepared from the native envelope protein or immunized with the envelope protein and the adjuvant Quil A.

Feline immunodeficiency virus (FIV) is a lentivirus that was first isolated from domestic cats which displayed clinical signs similar to those found in humans with AIDS (Pedersen et al., 1987). Like human and simian lentiviruses, FIV is T lymphotropic, causes a loss of CD4+ T cells, can infect macrophages and astrocytes, persists in infected cats and causes immunodeficiency in its natural host (Brunner & Pedersen, 1989; Ackley et al., 1990; Dow et al., 1990; Siebelink et al., 1990; Torten et al., 1991; Hoffmann-Fezer et al., 1992). Because of the similarities found between FIV infection in cats and human immunodeficiency virus (HIV) infection in humans, feline AIDS has been considered to be a useful small-animal model for the evaluation of strategies for the development of antiviral compounds and experimental vaccines to combat human AIDS.

The env gene-encoded surface (SU) and transmembrane (TM) glycoproteins of lentiviruses play a crucial role in the process of virus-cell attachment and fusion and serve as targets for neutralizing antibodies and cytotoxic T cells which are major components of antiviral immunity. Consequently, for the development of vaccines against FIV infection in cats, the incorporation of FIV glycoproteins presented in a form that stimulates both cell- and antibody-mediated immunity should be considered. It has been shown that immune-stimulating complexes (iscoms) induce both virus-neutralizing antibodies (Morein et al., 1984; Osterhaus et al., 1985; De Vries et al., 1988) and MHC class I-restricted CD8+ cytotoxic T lymphocytes (CTL) against retrovirus glycoproteins (Takahashi et al., 1990). Furthermore, we and others have recently documented that the iscom presentation form allows the endogenous processing which is necessary for both MHC class I- and class II-restricted recognition by CD8+ and CD4+ T cells, respectively (Heeg et al., 1991; Mowat et al., 1991; Van Binnendijk et al., 1992). Therefore, we have chosen the iscom structure as a basis for our strategy for the development of an FIV vaccine.

The incorporation of proteins into iscoms is largely facilitated by the presence of regions which allow hydrophobic interaction with the iscom matrix (Claassen & Osterhaus, 1992). Since the TM protein of FIV env is highly hydrophobic and the non-covalently bound TM and SU proteins readily dissociate, our strategy was to incorporate an FIV env protein from which the cleavage site between the SU and TM proteins had been deleted. We generated a recombinant vaccinia virus (rVV) that
expresses an FIV env protein from which this cleavage site had been deleted in order to facilitate incorporation of the SU protein in the iscom matrix.

The FIV env gene (2.6 kb) encoding the env precursor protein was amplified by PCR. Bone marrow-derived DNA, obtained from an FIV-infected cat was used as a source of FIV proviral DNA (Siebelink et al., 1992). The oligonucleotides for PCR amplification were synthesized on an Applied Biosystems DNA synthesizer. They were based on the nucleotide sequence of the Petaluma strain of FIV (Talbott et al., 1989): 5' GGCAAGTTGCAACTCATATTATC 3' and 5' GCACAATAAGAATGCTGAGCAG 3' (prime from the 5' end), 5' CTCAGCACAAGATCTATCTCCC 3' and 5' GACATACCTTCCTCAAA-GGG 3' (prime from the 3' end). The amplified fragment was cloned into the HindIII site of the cloning vector pBluescript II SK(+) (Stratagene). The FIV env gene was excised from the pBluescript construct (pB/env), with the restriction enzymes XhoI and SmaI, and cloned into the Sall- and Smal-digested plasmid pSC65 (S. Chakrabarti & B. Moss, unpublished results). This plasmid contained vaccinia virus thymidine kinase (TK) sequences flanking the cloning site, a synthetic early/late vaccinia virus promoter and the lacZ gene under control of the vaccinia virus 7.5 K promoter. The resulting plasmid was designated pGR657. Plasmids were screened by restriction endonuclease analysis and DNA hybridization using gel-purified env gene as a labelled probe (ECL; Amersham) and were grown by standard procedures (Sambrook et al., 1989). For the construction of an rVV that expresses the env precursor protein without the cleavage site between the SU and TM proteins, the nucleotide sequence at positions 1822 to 1833, coding for the potential cleavage site RRKR, was deleted. To this end, 3 µg of pBS/env was used as template DNA for amplification by PCR using the primers 5'-del (5' GGCAAGTTGCAACTCATATTATC 3'; nt 1800 to 1856 from which the nucleotide sequence at positions 1822 to 1833 were deleted) and 3' BgII (5' GGAGGTAGATCTTTTGTGTTATACC 3'; nt 2204 to 2227), to obtain fragment 5'-del/3'-BgII. Using primer pair 3'-del (reverse complementary to 5'-del) and 5'-NsiI (5' GACCTATATTATGCAATTATGATCAAATTTTGATGGAATATACATGTCGTGCTATGCATTCTATGGTTGGGC 3'; nt 1800 to 1856 from which nt 1822 to 1833 were deleted) and 3' BgII (5' GGAGGTAGATCTTTTGTGTTATACC 3'; nt 2204 to 2227), to obtain fragment 5'-del/3'-BgII. Using primer pair 3'-del (reverse complementary to 5'-del) and 5'-NsiI (5' GACCTATATTATGCAATTATGATCAAATTTTGATGGAATATACATGTCGTGCTATGCATTCTATGGTTGGGC 3'; nt 1534 to 1567), fragment 5'-NsiI/3'-del fragment was obtained. To obtain the 5'-NsiI/3'-BgII fragment with the deletion, a PCR was performed using the 5'-NsiI and 3'-BgII primers and 500 ng of the fragments 5'-NsiI/3'-del and 5'-del/3'-BgII as template. The PCR-derived 5'-NsiI/3'-BgII fragment was purified by preparative gel electrophoresis, digested with NsiI and BgII and cloned into NsiI/BgII-digested pGR657. The resulting plasmid was designated pGR657X15. The presence of the deletion was confirmed by determination of the nucleotide sequence in this region using dideoxy-nucleotide chain termination sequencing. Subsequently rVV were generated as previously described (Mackett et al., 1984) by homologous recombination with vaccinia virus (WR strain), which was obtained originally from the ATCC. Recombinant virus plaques were visualized by their blue colour as a result of the co-expression of the lacZ gene and overlay with X-gal (Chakrabarti et al., 1985). The viruses were plaque-purified three times and stocks of vGR657 and vGR657X15 were grown in HeLa cells.

The FIV proteins expressed by vGR657 and vGR657X15 were analysed by pulse-labelling in a radioimmunoprecipitation assay (RIPA) and Western blot analysis. For RIPA HeLa cells were infected with vGR657, vGR657X15 or vSC65 (control rVV made with pSC65) at an m.o.i. of 30 p.f.u. per cell. At 4 h post-infection the cells were incubated in methionine- and cysteine-free medium for 30 min after which they were pulse-labelled for 30 min with [35S]methionine and [35S]cysteine (200 µCi/ml), followed by different chase periods (0, 2 and 24 h). FIV-specific proteins were immunoprecipitated from both culture supernatants and cell lysates with polyclonal anti-FIV antibody (serum from the naturally infected cat Adam 19; Siebelink et al., 1992) and the monoclonal antibody (MAB) 6-13-12, specific for FIV TM protein (see below).

For the generation of this MAB, BALB/c mice were immunized with iscoms in which the FIV envelope proteins expressed by rVV vGR657 were incorporated. For incorporation in iscoms, lysates of vGR657-infected cells were solubilized in PBS containing 2% MEGA-10 (Boehringer Mannheim). Subsequently, glycoproteins were purified by affinity chromatography using lentil lectin–Sepharose (Pharmacia). The purified protein was mixed with the lipids cholesterol and phosphatidylethanolamine (Sigma) and Quil A (Spikoside; ISCOTEC) at a ratio of 1:1:5 (w/w) and after ultrasonication for 30 s were incubated for 1 h at room temperature in the presence of 0.2% MEGA-10. The mixture was then dialysed against PBS for 16 h at room temperature followed by dialysis against PBS for 24 h at 4°C. The resultant iscoms were analysed by electron microscopy, revealing the typical cage-like structure, and also by SDS–PAGE followed by Western blot analysis. This provided evidence for the predominant incorporation of the TM protein (not shown). BALB/c mice were immunized with these iscoms and hybridomas were generated according to standard procedures (Osterhaus et al., 1981). For the identification of hybridomas producing antibodies reactive with FIV env proteins an FIV env-specific ELISA was used (Rimmelzwaan et al., 1994).

MAB 6-13-12, selected for these studies, proved to be specific for the TM protein of FIV as demonstrated by its
Fig. 1. Pulse-chase analysis of the generation of FIV proteins after rVV infection of HeLa cells. After infection with rVV vSC65, vGR657 or vGR657X15, cells were labelled with [35S]methionine and [35S]cysteine. At 0, 2 and 24 h after labelling (lanes 1, 2 and 3 respectively) FIV env proteins in cell lysates and culture medium were visualized by immune precipitation and PAGE using (a) MAb 6-13-12 and (b) serum from FIV-seropositive cat Adam 19.
reactivity with FIV env proteins in RIPA (Fig. 1a) and in the immunostaining of Western blots (Fig. 2a and 3a).

Directly after pulse labelling of cells infected with vGR657 two FIV proteins with MrS of 150K and 130K were observed in the cell lysates by immunoprecipitation with the serum of cat Adam 19. After a chase period of 2 h the 150K protein had disappeared and only the 130K protein remained. Twenty-four h after labelling a smear of protein with a mean Mr of 32K and a 95K protein were visualized in addition to the 130K protein. In the supernatant of the culture infected with vGR657 a 95K protein only was observed (Fig. 1b).

These results show that in cells infected with rVV vGR657 a 150K FIV precursor envelope protein is synthesized which is then processed into a 130K protein. This protein is subsequently cleaved into a 95K protein that is released into the culture supernatant and most likely represents the SU protein and a 32K protein which most likely represents the TM protein. Thus the processing of FIV glycoproteins expressed by rVV appears to be similar to the processing of FIV glycoproteins in persistently infected Crandell feline kidney (CrFK) cells as described by Stephens et al. (1991).

In the cell lysate fraction of cells infected with rVV vGR657X15 FIV proteins with MrS of 150K and 130K were also present directly after pulse labelling. After a chase period of 2 h the 130K protein proved to be predominantly precipitated and after a chase period of 24 h this was the only FIV protein observed. No FIV protein was precipitated from the supernatants of the cultures infected with rVV vGR657X15 (Fig. 1b). These data suggest that in cells infected with vGR657X15, an FIV precursor envelope protein with an Mr of 150K was synthesized and that this protein was processed into a 130K protein that is not cleaved like the native protein. Immunoprecipitation studies with MAb 6-13-12 using the same samples confirmed this suggestion. With this MAb, the 150K, 130K and 32K proteins, but not the 95K protein, were immunoprecipitated from lysates of cells infected with rVV vGR657 (Fig. 1a). Proteins with MrS of 150K and 130K were precipitated from cells infected with rVV vGR657X15. No 32K protein was precipitated from these lysates, indicating that the 130K protein expressed by vGR657X15 is not cleaved.

When lysates of rVV-infected BHK cells were tested for the presence of FIV proteins by Western blot analysis, the 130K protein could be detected in vGR657X15-infected cells but not in VSC65- or vGR657-infected cells. In vGR657-infected cells the TM protein could be detected but it was not detected in vSC65- or vGR657X15-infected cells (Fig. 2), which confirms the findings of the pulse labelling experiment.

Incorporation studies with lentil lectin-purified extracts from BHK cells infected with the respective rVVs showed that iscoms prepared from purified extracts of rVV vGR657X15-infected cells (as described above) predominantly incorporated the whole uncleaved 130K protein, in addition to minor amounts of TM protein (32K protein), as shown in Western blot analysis using MAb 6-13-12 (Fig. 3a) or serum from seropositive cat Adam 19 (Fig. 3b). We speculated that the presence of the 32K protein was the result of a putative secondary cleavage site and that this protein was found now as a result of the concentration of the hydrophobic proteins during the process of iscom formation. In contrast, iscoms prepared from similarly purified extracts of vGR657-infected cells incorporated no 130K protein,
but did incorporate the TM protein and a 64K protein that was speculated to represent a dimer of the TM protein (Fig. 3). The 130K protein incorporated in the iscom matrix is of particular interest as an immunogen since it may be expected to contain the majority of the T and B cell epitopes of the native envelope proteins, although it cannot be ruled out that the conformation of this protein has been changed by the deletion of the cleavage site. However, it was recently shown that an rVV expressed gp160 of HIV-1 from which the cleavage site had been deleted still bound soluble CD4 (Earl et al., 1991) or membrane-bound CD4 (Kieny et al., 1988), indicating that at least the conformation-dependent CD4-binding site had retained its conformation in this protein.

The immunogenicity of these candidate FIV vaccines was tested in cats. Three groups of six cats were immunized subcutaneously twice with a 4 week interval. Each vaccine dose contained either 10 µg vGR657 iscoms, 10 µg vGR657X15 iscoms or 10 µg vGR657X15 as soluble protein mixed with 20 µg Quil A. A fourth group was immunized twice in the same way with 10 µg of control iscoms prepared with cleavage site-deleted simian immunodeficiency virus (SIVmac) envelope protein, essentially in the same way as described for vGR657X15 iscoms. Serum samples collected at day 0, 28 and 56 post-immunization were tested in serial twofold dilutions for the presence of antibodies against two synthetic peptides in an ELISA. The peptides were a kind gift from R. van Herwijnen, European Veterinary Laboratory, Woerden, The Netherlands. The first peptide contained the linear virus neutralization site within variable region 3 and comprises amino acid residues 396 to 412 of the surface protein of the FIV Petaluma strain (SU peptide) (Lombardi et al., 1993), and the second peptide comprised a B cell epitope between amino acid positions 824 and 848 of the transmembrane protein of the same FIV strain (TM peptide) (Avrameas et al., 1992). Antibody titres of serum samples were calculated by determining the highest dilution at which the A450 was still higher than three times the A50 of the serum sample from the same cat taken prior to immunization.

All six cats immunized with vGR657X15 iscoms showed serum antibody titres ranging from 50 to 200 to both the SU and TM peptides 56 days post-immunization (Fig. 4). Five of six cats immunized with vGR657 iscoms showed serum antibody titres ranging from 50 to 200 to the SU peptide, whereas no serum antibodies to the TM peptide could be demonstrated. This is surprising since these iscoms contained TM protein and no detectable SU protein. One explanation for this observation may be that the conformation of the epitope presented by the TM peptide was not properly exposed in vGR657 iscoms. Apparently the vGR657 iscoms still contained enough residual SU protein to induce an SU-specific antibody response. Only three of the six cats immunized with vGR657X15 soluble protein mixed with Quil A developed serum antibodies to the SU peptide with titres ranging from 100 to 200, whereas no antibodies to the TM peptide could be detected. None of the cats immunized with the control SIV envelope iscoms developed detectable antibodies to either of the peptides. Thus the cleavage site-deleted envelope protein incorporated into the iscom matrix proved to be most efficient in inducing antibodies to both the SU and TM proteins.

The antibody responses will be analysed further to evaluate whether antibodies to other epitopes or virus-neutralizing antibodies have been induced. The evaluation of vGR657X15 iscoms as a potential candidate FIV vaccine is the subject of our present studies.

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