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Visna/maedi virus Env protein expressed by a vaccinia virus recombinant induces cell-to-cell fusion in cells of different origins in the apparent absence of Env cleavage: role of glycosylation and of proteoglycans

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Summary. The *in vivo* productive infection by the ovine Visna/maedi lentivirus (VISNA) is restricted to cells of the monocyte/macrophage lineage. The basis for this restriction is not understood. Although the VISNA envelope (Env) glycoprotein is the main target for virus neutralization, studies on the role of this protein in virus infection are limited. A vaccinia virus recombinant (VV-*env*-MV) containing the entire VISNA *env* sequence was generated and shown to produce in infected cells a protein of about 165 kDa (referred to as gp150). During VV-*env*-MV infection, expression of *env* caused extensive cell-to-cell fusion in cell lines of different origins. Pulse-chase and Western blot analyses revealed that gp150 is not cleaved in VV-*env*-MV infected cells. The glycoprotein gp150 formed oligomers held by disulfide bonding. Cell-to-cell fusion was prevented in the presence of the inhibitor of glycosilation, tunicamycin, but it was markedly enhanced by an inhibitor of proteoglycan synthesis, β-D-xyloside. These findings showed that the receptor for VISNA Env is widely distributed within cells, that fusion-from-within of cells can occur in the apparent absence of proteolytic cleavage of gp150, and that fusion require a glycosylated Env but not the addition of proteoglycan chains at the cell surface. This recombinant virus could have utility as a potential vaccine against VISNA.

Introduction

Visna/maedi virus (VISNA) is an ovine lentivirus that causes a progressive and degenerative disease affecting the mammary glands, lungs, brains, joints and the

immune system [4, 34, 36]. The steep rise of the VISNA infection in sheep, together with the absence of effective therapeutic treatments, prompted to the development of sensitive diagnostic methods [38] and effective vaccines that could confer long-term immunity against this virus or inhibit disease development. In lentiviruses, the envelope glycoprotein (Env) is the major antigenic determinant, harboring the targets for most neutralizing antibodies [17, 21, 42] and several CTL epitopes [9, 27, 40]. Thus, immunogens expressing or containing the Env protein could be good candidates for vaccine development. The complete nucleotide sequence of the *env* region from several ovine VISNA strains has been determined and found to code for an Env protein consisting of nearly 1000 amino acids [5, 33, 39]. The Env protein is synthesized in the course of virus infection as a high molecular weight precursor (ranging from 150 to 170 kDa according to different authors) which is heavily glycosylated and proteolytically cleaved to generate the surface gp135 and the transmembrane gp46 glycoproteins [6, 43]. Different fragments of VISNA Env have been produced as recombinant proteins in different expression systems, including the bacterial pGEX system [23, 24], the yeast Ty system [6] and baculovirus [7, 24]. However, the expression of the entire Env protein in mammalian cells in the absence of VISNA infection has not been reported.

Recombinants of vaccinia virus (VV) have been widely used to express foreign proteins [32, 35]. In general, polypeptides synthesized by VV vectors are biologically active and are processed and transported according to the primary structure of the protein. This allows for the structural and functional analyses of recombinant proteins expressed by this system. In addition, it has been shown that VV recombinants elicit a protective immune response against a variety of pathogens [32, 35]. Based on these findings, it has been suggested that VV recombinants could serve as live vaccines against infectious agents in humans and animals.

As a preliminary step towards the generation of a vaccine against VISNA, we have constructed a recombinant VV harboring the complete VISNA *env* sequence and analyzed the properties of the Env protein in cells infected by this recombinant virus.

Materials and methods

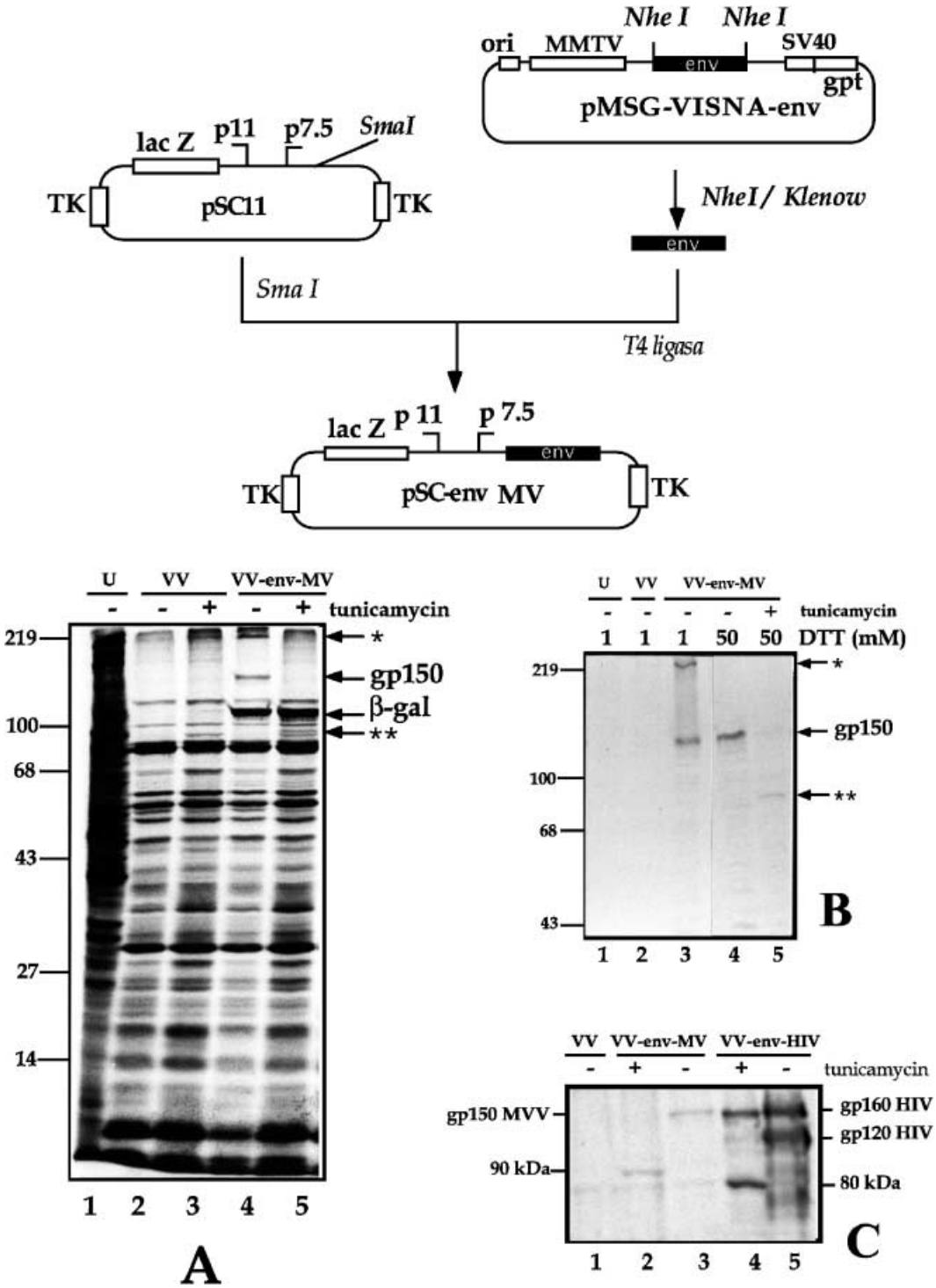
Cells and virus recombinants

HeLa human cells and monkey kidney BSC-40 cells were grown in DME medium containing 10% newborn calf serum (NCS) and antibiotics. Cultures of primary chick embryo fibroblasts (CEF), mouse fibroblasts (3T3 and L), sheep fibroblasts, 293 T human fibroblast cells, and rabbit kidney cells (RK13) were grown in DME with 10% fetal calf serum. The recombinant VV containing the entire coding sequence for the VISNA Env glycoprotein from the EV1 [5] strain (VV-*env*-MV) was generated following standard procedures previously described [37], using a plasmid harboring the full length of VISNA *env*, pMSG-VISNA-*env* (kindly provided by Dr. R. G. Dalziel, University of Edinburgh) and the VV transfer vector pSC11 [8]. A VV insertion vector containing the VISNA *env* coding sequence was constructed according to the strategy depicted in Fig. 1 (top), and the resulting plasmid, pSC-*env*-MV, was introduced by homologous recombination into the thymidide kinase (TK) region of the VV genome to generate the recombinant virus VV-*env*-MV.

Results

Expression of the entire VISNA Env protein by a vaccinia virus recombinant (rVV-env-MV)

To identify and characterize the VISNA Env protein, African green monkey kidney (BSC-40) cells were infected at a multiplicity of infection of 5 PFU/cell with either the recombinant VV-*env*-MV or the parental VV (strain WR) in the presence or absence of the glycosylation inhibitor tunicamycin (5 μg/ml). Infected cells were metabolically labeled with [^{35}S]-methionine (10 μCi/ml) from 6 to 24 h.p.i. and cell extracts were analyzed by SDS-PAGE. As shown in Fig. 1, panel A, in addition to β-galactosidase two other polypeptides were observed in extracts from untreated VV-*env*-MV-infected cells (lane 4) which were not present in extracts from cells infected with the parental VV (lane 2). One of the polypeptides had an apparent molecular mass of about 150 kDa and the other was a very slow (> 200 kDa) migrating product (denoted with a single asterisk). These two products could correspond to the monomeric and oligomeric forms of the Env precursor, respectively. In contrast, in cells infected by VV-*env*-MV in the presence of tunicamycin (lane 5), both polypeptides were absent. Instead, a product with an apparent molecular mass of about 90 kDa was observed (denoted with two asterisks), which could correspond to the unglycosylated form of Env, as previously proposed [33, 43]. To confirm that these polypeptides corresponded to Env, the same samples were analyzed by immunoblot using an anti-gp135 serum (Maeditect 100, Central Veterinary Laboratory, UK). The result is shown in Fig. 1, panel B. In extracts from VV-*env*-MV infected cells (lane 3), prepared in the presence of a low concentration (1 mM) of the reducing agent dithiothreitol (DTT), the anti-gp135 antibody specifically recognized two polypeptides, one of about 150 kDa and the other of a higher molecular weight (> 200 kDa, denoted with a single asterisk), being both absent in extracts from uninfected cells (lane 1) and from cells infected with the parental VV (lane 2). The size and reactivity with anti-VISNA Env antibody, indicate that the 150 kDa protein corresponds to the Env precursor, and hence, it will be referred to as gp150 [43]. Upon treatment of extracts from VV-*env*-MV infected cells with a higher concentration of DTT (50 mM), an increase in the reactivity of the gp150 band was observed, that was concomitant with the complete disappearance of the larger polypeptide (lane 4), indicating that the slow-migrating band corresponded to an oligomeric form of gp150. In the presence of tunicamycin (lane 5), an immunoreactive product of about 90 kDa was observed, suggesting that it corresponds to the unglycosylated form of Env. These findings are compatible with those in previous work on VISNA and the closely related caprine arthritis-encephalitis virus (CAEV) infection, showing that the non glycosylated Env precursor is a polypeptide of about 90 kDa [11, 43]. Moreover, based on the aminoacid sequence, the predicted molecular size of VISNA Env is 103 kDa [33]. To define more precisely the sizes of the VISNA Env glycosylated and non glycosylated products expressed by VV-*env*-MV, extracts from cells infected with VV-*env*-MV in the presence and absence of tunicamycin were fractionated by SDS-PAGE in parallel with extracts from BSC-40 cells infected with a VV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tunicamycin
219
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1 2 3 4 5
A
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VV
VV-env-MV
VV-env-HIV
gp150 MVV
90 kDa
gp160 HIV
gp120 HIV
80 kDa
C

recombinant that harbors the *env* gene from the human immunodeficiency virus (HIV-1). This virus, referred to in this paper as VV-*env*-HIV (VVenv in reference [37]), produces the well characterized gp160 precursor and its cleavage products gp120 and gp41. Proteins were transferred to a nitrocellulose membrane and reacted first with the anti-gp135 antibody to visualize the VISNA glycosylated and unglycosylated Env products, and then reacted with an antibody specific for the HIV-1 Env protein to detect the precursor gp160 and its cleavage product gp120, as well as the unglycosylated 80 kDa polypeptide. Figure 1C shows that the size of the VISNA Env precursor produced in the absence of tunicamycin (lane 3) was similar to the HIV-1 precursor produced under the same conditions (lane 5). However, the unglycosylated VISNA Env shows a higher molecular weight than that from HIV-1 (compare lanes 2 and 4). Neither of the two sera recognized a protein in extracts from cells infected with the parental VV (lane 1). The result of Fig. 1C indicates that in BSC-40 cells infected with VV-*env*-MV the VISNA Env product accumulates in the form of a glycosylated precursor, with an apparent molecular mass close to 160 kDa of HIV-1 *env*.

Fig. 1. Expression of the VISNA Env protein in VV-*env*-MV infected cells. Upper panel. Scheme of the cloning strategy for the construction of the VV insertion vector containing the VISNA *env* gene. A *Nhe*I DNA fragment containing the entire VISNA *env* gene was isolated by agarose gel electrophoresis from the pMSG-VISNA-*env* plasmid. This DNA fragment was blunt ended by treatment with the *Klenow* enzyme and cloned into the *Sma*I site of the VV insertion vector pSC11 to produce the plasmid pSC-*env*-MV, which contains the *env* coding sequence under the control of the p7.5 VV early–late promoter and the β-Galactosidase gene under the control of the p11 VV late promoter. **A.** Metabolic labeling. Monolayers of BSC-40 cells were infected (5 PFU/cell) with either the parental VV (*2* and *3*) or the recombinant VV-*env*-MV (*4* and *5*) in the presence (+) or absence (−) of the glycosylation inhibitor tunicamycin. Uninfected (*1*) and infected cells were metabolically labeled with [^{35}S]-methionine (10 μ Ci/ml) from 6 to 24 h.p.i. Cells were harvested and labeled proteins were analyzed by 10% SDS-PAGE and autoradiography. **B.** Western-blot analysis. Extracts from uninfected cells (*1*) and cells infected with the parental virus VV (*2*) or the recombinant VV-*env*-MV in the absence (*3* and *4*) or presence (*5*) of tunicamycin were analyzed by SDS-PAGE (7%). Extracts were treated with 1 or 50 mM DTT, as indicated on the top of the figure. Proteins were transferred to a nitrocellulose membrane and allowed to react with an anti-gp135 antibody (Maeditect 100, Central Veterinary Laboratory, UK). Immunoreactive bands were detected after incubation with a peroxidase-conjugated secondary antibody (Organon Teknika). **C.** Comparative analysis of VISNA Env and HIV-1 Env. Monolayers of BSC-40 cells were infected (5 PFU/cell) with either the parental VV (*1*), VV-*env*-MV (*2* and *3*) or a VV recombinant expressing the entire HIV-1 Env (*4* and *5*). Infections were performed in the presence (+) or absence (−) of tunicamycin. Proteins were transferred to a nitrocellulose membrane and reacted with anti-gp135 antibody. After incubation with a peroxidase-conjugated secondary antibody immunoreactive bands were detected, and the membrane was then incubated with a polyclonal antiserum directed against HIV-1 gp120, and HIV-1 specific proteins were visualized after reactivity with peroxidase-conjugated secondary antibody. The positions of the HIV-1 gp160, gp120 and unglycosylated 80 kDa precursor are indicated on the right side of panel **C**. Molecular mass markers (in kilodaltons) are represented to the left of panels **A** and **B**

VISNA Env is apparently not cleaved in cells infected with rVV-env-MV

While during VISNA infection Env is cleaved to produce the surface gp135 and the transmembrane gp46 glycoproteins [6, 43], it was of interest to know if, as suggested from the experiments shown in Fig. 1, Env generated from a VV recombinant was indeed uncleaved. Thus, we performed pulse-chase labeling with cells infected with VV recombinants that express either Env from VISNA or Env from HIV-1; thereafter, cell extracts were immunoprecipitated with Env specific antibodies, the proteins were fractionated by SDS-PAGE and the results of the autoradiograms were compared between the two envelope proteins. As shown in Fig. 2, in cells infected with VV-*env*-MV the main band visualized after a 10 min pulse labeling was gp150 (lane 1). A band of similar size and intensity was observed after immunoprecipitation from cells chased for a 16 h period (lane 2). When immunoprecipitation was performed from the extracellular medium of these infected cells we failed to observe any protein band (lane 4). If tunicamycin was added to the infected cells, the main band had now a molecular mass of about 90 kDa (lane 3). However, as expected, in cells infected with VV-*env*-HIV after a 10 min pulse the precursor gp160 was observed (lane 5), but the intensity of this band decreased after the 16 h chase period, and a concomitant appearance of the mature gp120 protein was observed (lane 6). Immunoprecipitation with anti-gp160 antibodies from the extracellular medium of these cells clearly show that the processed gp120 was efficiently secreted (lane 8). After tunicamycin treatment,

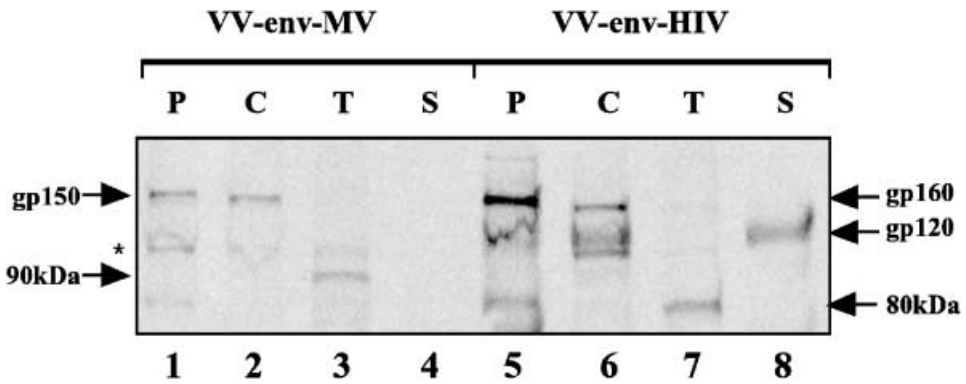

Fig. 2. Pulse-chase experiments revealed that the Env protein produced from VV-*env*-MV is not cleaved. BSC-40 cells were infected with VV-*env*-MV or with VV-*env*-HIV (5 pfu/cell) and at 6 h.p.i cells were pulse-labeled with [^{35}S]-methionine for 10 min. Thereafter, cells were treated with a 10-fold excess of cold methionine and cell supernatants and whole cells were collected after 16 h of chase. Whole cells were lysed for 30 min in buffer (20 mM Tris-HCl, pH 8.0, 80 mM NaCl, 20 mM EDTA, 1% NP-40) and clarified by centrifugation. The VISNA and HIV Env proteins were immunoprecipitated with specific antibodies from cell supernatants and cell lysates and analyzed by SDS-PAGE and autoradiography. Cells infected with VV-*env*-MV (*1* to *4*); cells infected with VV-*env*-HIV (*5* to *8*). The abreviations are: *P*, whole cells collected after the 10 min pulse labeling; *C*, cells collected after the 16 h chase; *T*, cells infected in the presence of tunicamycin and collected after the pulse; *S*, cell supernatants collected after the 16 h chase. The size of the different glycoproteins is indicated

the main protein band has shifted to a molecular mass of about 80 kDa (lane 7). An unspecific band of about 110 kDa (labeled with an asterisk) was observed in several lanes from both VV-*env*-MV and VV-*env*-HIV infected cells. The findings of Fig. 2 revealed that in BSC-40 cells infected with VV recombinants, Env from HIV is proteolytically cleaved to form gp120 while Env from VISNA is apparently not processed. Moreover, by direct comparison of sizes, Env from VISNA has a molecular mass of about 165 kDa, slightly higher than the well characterized 160 kDa Env from HIV-1. For simplicity, we referred throughout this work the uncleaved Env from VISNA as gp150.

A single protein of gp150 is produced in ovine cells infected with rVV-env-MV

To further characterize if the lack in processing of VISNA Env protein expressed by VV-*env*-MV is related to the expression of the protein in cells non-permissive for VISNA, we examined protein expression in the natural host of VISNA, the ovine cells, and compared the pattern with BSC-40 cells. Thus, ovine fibroblasts or BSC-40 cells were infected with VV-*env*-MV, and at 12 and 24 h post infection cell extracts were analyzed by SDS-PAGE gel and Env revealed by immuno blot with a gp135 antibody. As shown in Fig. 3, at both times post-infection a single immune reactive band corresponding to gp150, was observed both in ovine and monkey cells infected with VV-*env*-MV. These findings revealed that the lack of

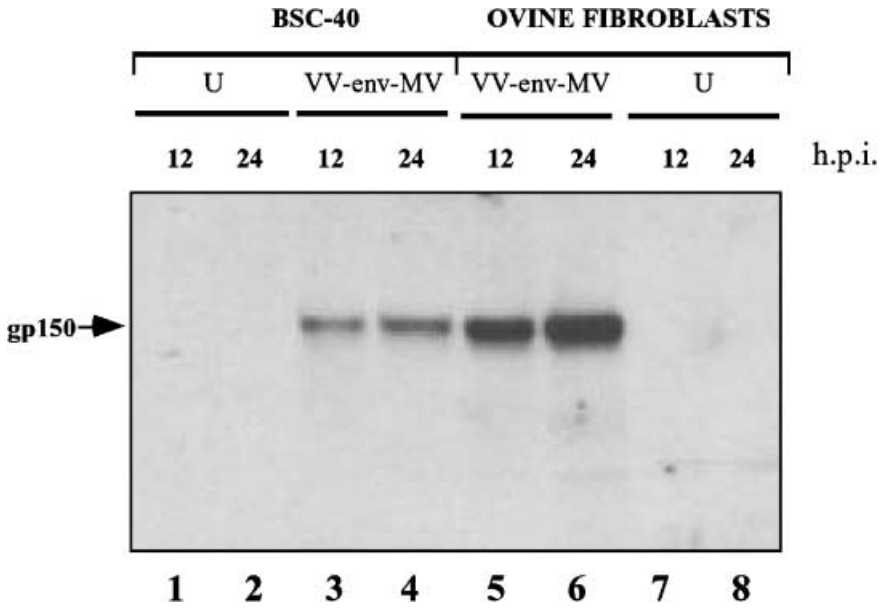

Fig. 3. The Env protein produced by VV-*env*-MV in ovine fibroblasts has similar size as in non VISNA permissive cells. BSC-40 cells and ovine cells were infected with VV-*env*-MV (5 pfu/cell) and at 12 and 24 h.p.i, cell extracts were fractionated by SDS-PAGE, blotted and reacted with specific anti-gp135 antibody. The same immunoreactive gp150 was observed for the different cell types, with no evidence for cleavage

processing of Env in monkey cells is not due to the cell origin, as the natural host, ovine cells, generate the same molecular mass protein.

VISNA-Env produced by rVV-env-MV is antigenically related to the protein from VISNA-infected cells

Next, we asked whether the recombinant protein produced by VV-*env*-MV was antigenically related to the protein produced in the natural host during infection with VISNA. Thus, extracts from VV-*env*-MV infected cells were fractionated by SDS-PAGE and blots were reacted with different antibodies. As shown in Fig. 4, serum from infected sheep recognized the gp150 precursor (panel B, lane 1), but not in extracts from cells infected with non-recombinant VV (panel B, lane 2). With anti-gp135 serum, the only recognized protein is gp150 (panel A, lane 1). There was no reactivity of serum from an uninfected sheep with gp150 (panel C, lane 1). As shown in panels B and C, sera from uninfected or VISNA-infected sheep recognized VV proteins, probably because of natural infection with a sheep poxvirus. The findings of Fig. 4 provided additional information for a lack of processing of gp150 in cells infected with VV-*env*-MV.

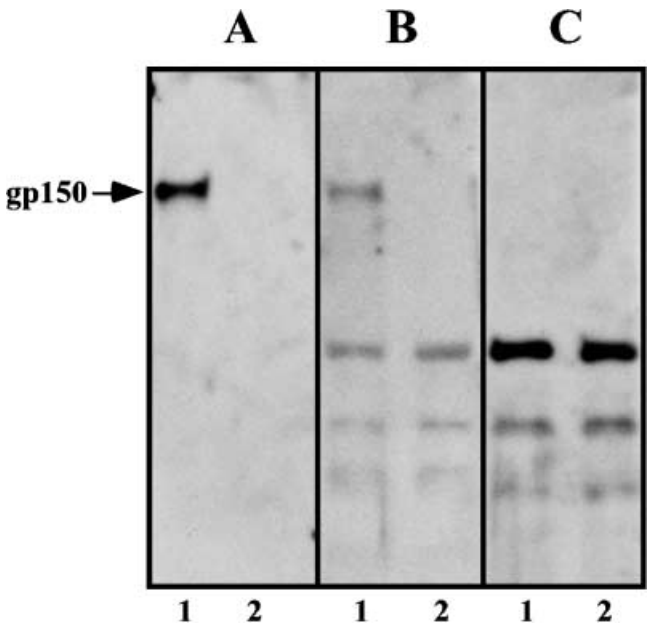

Fig. 4. Serum from VISNA-infected sheep reacted with gp150 expressed from VV-*env*-MV. Extracts from BSC-40 cells infected with VV-*env*-MV (*1*) or with the parental VV (*2*) were fractionated by SDS-PAGE, proteins transferred to a nitrocellulose paper and strips containing both types of cell extracts were probed with sera from different origins. **A** Anti-gp135 antibody. **B** Serum from VISNA infected sheep. **C** Serum from a sheep that was not infected with VISNA. The same band of gp150 was observed in panels **A** and **B** but not in **C**. The lower size reactive bands appearing in panels **B** and **C** are probably due to a previous poxvirus infection of the sheep

Cells of different origins infected with rVV-env-MV trigger extensive cell-to-cell fusion

In permissive cells infected *in vitro*, such as fibro-epithelial cells of goat synovial membrane, and fibroblasts such as those of sheep choroid plexus, VISNA may cause an extensive cytopathic effect characterized by formation of syncytia [1, 10, 15, 41]. This fusogenic phenomenon is mediated by the Env protein present in the surface of infected cells, suggesting the use of this protein for vaccination purposes [25]. It has been shown that a hydrophobic peptide of 24 aminoacids from the N-terminus of the Env transmembrane domain induces fusion when added to primary goat synovial membrane cell cultures [14]. In order to determine whether the Env protein expressed by the VV-*env*-MV recombinant virus was capable of inducing cell-to-cell fusion, we used cells of different origins. Cultures of primary chick embryo fibroblasts (CEF), mouse fibroblasts (3T3 and L), sheep fibroblasts, 293 T human fibroblast cells, human epithelial cells (HeLa), monkey epithelial cells (BSC-40) and rabbit kidney cells (RK13) were infected with VV-*env*-MV (5 PFU/cell), in the presence or absence of an inhibitor of glycosylation (tunicamycin) and the formation of syncytia was evaluated by phase contrast microscopy at 24 h.p.i. The results are summarized in Table 1 and representative findings are shown in Fig. 5. Multinucleated giant cells were present in all cell lines tested when infections were performed in the absence of tunicamycin, but different degrees of cell fusion were observed. In BSC40 cells infected with the recombinant VV-*env*-MV in the presence of tunicamycin, or in cells infected with the parental VV, syncytium formation was not observed, although a cytopathic effect characteristic of VV infection was evident. These results of Fig. 5 showed that, in the absence of other VISNA factors, the intracellularly produced VISNA Env protein can mediate cell-to-cell fusion, and that this property requires protein glycosylation. It has been recently described that treatment with an inhibitor of glycosaminoglycan (GAG) synthesis significantly reduces VISNA production [3].

Table 1. Fusogenic activity of VISNA env glycoprotein in different cell lines

Cell lines	Extent of cell–cell fusion (*)
Human Hela	+
Monkey BSC40	+++
Rabbit RK13	+++
Chick embryo fibroblast	++
Mouse 3T3	++++
Mouse L929	++++
Hamster BHK-21	++

(*) The relative extent of syncytium formation was established at 24 h.p.i. after examination of the VV-*env*-MV infected cultures (5 pfu/cell) under the microscope; +, represents 25% fusion

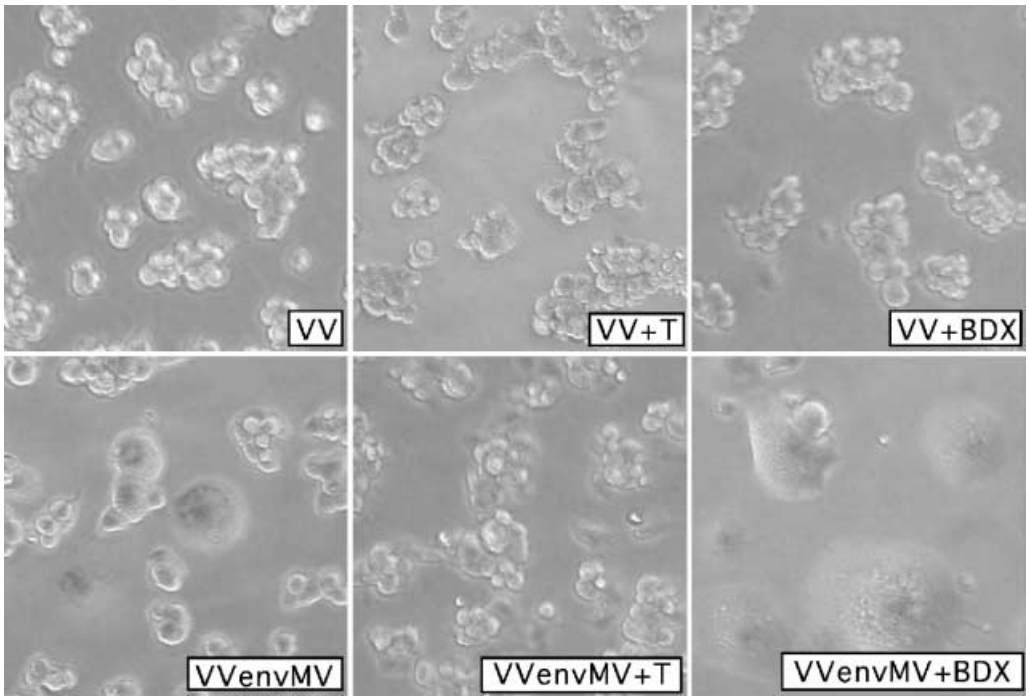

Fig. 5. Expression of Env protein in BSC40 cells infected with VV-*env*-MV induced cell-to-cell fusion and this effect is blocked by inhibition of glycosylation but is enhanced by blocking GAG chain synthesis. The fusogenic properties of the VISNA Env protein were analyzed in monolayers of BSC-40 cells. Cells were infected (5 PFU/cell) with the parental VV or with the recombinant VV-*env*-MV in the presence or in the absence of tunicamycin (*T*) or of the inhibitor of proteoglycan synthesis, β-D-xyloside (*BDX*). Cell cultures were photographed at 24 h.p.i. on a Zeiss microscope

Thus, it was of interest to determine whether the fusogenic activity of Env expressed by VV-*env*-MV could be affected by pretreatment of cells with the reagent β-D-xyloside that inhibits the addition of GAG to proteoglycans. To our surprise, the extent of Env mediated fusion was significantly increased by pretreatment with β-D-xyloside (Fig. 5). Under these conditions syncytium formation was not observed in cells infected with the parental VV virus. On the other hand, it has also been previously reported that expression of Env by 293 cells did not cause cell-to-cell fusion unless they were co-cultured with cells from a different cell line (BHK21) expressing Env [28]. We then wished to determine whether pretreatment of 293 cells with β-D-xyloside could facilitate Env mediated fusion. As shown in Fig. 6 in the absence of β-D-xyloside a cytopathic effect similar to that induced by the parental VV virus but not fusion was observed in cells infected with VV-*env*-MV similarly to what happens in cells infected in the presence of tunycamicin. However, extensive syncytium formation was observed when cells had been pretreated with β-D-xyloside before infection with VV-*env*-MV. Again, this fusogenic activity was associated to the expression of Env protein, since β-D-xyloside pretreated cells infected with the parental VV did not show any fusogenic phenotype. Analysis by western-blot showed that the Env protein expressed in cells

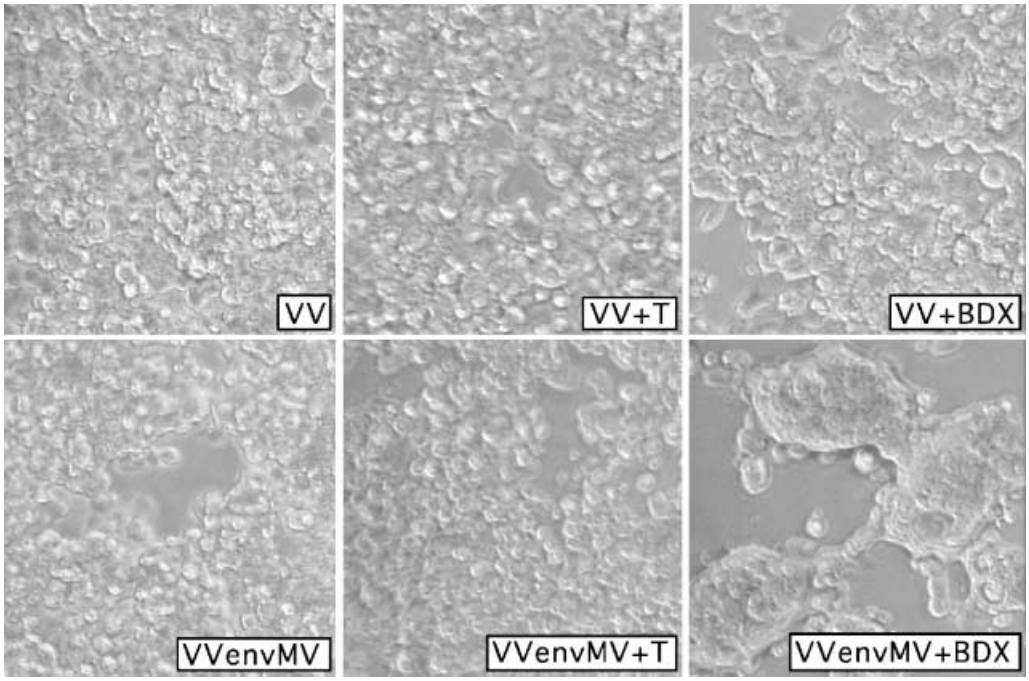

Fig. 6. Pretreatment with the GAG chain synthesis inhibitor **β**-D-xyloside before infection with VV-*env*-MV changes the phenotype of 293 infected cells from non-fusogenic to fusogenic. 293 cells were infected (5 PFU/cell) with the parental VV or with the recombinant VV-*env*-MV in the presence or in the absence of tunicamycin (*T*) or of the inhibitor of proteoglycan synthesis, β-D-xyloside (*BDX*). Cell cultures were photographed at 24 h.p.i. on a Zeiss microscope

pretreated with β-D-xyloside was indistinguishable to that produced in untreated cells (data not shown).

In the case of HIV-1, it has been shown that the expression of the Env protein on the surface of the cells is able to cause apoptosis of cells bearing the CD4 molecule, even in the absence of other viral factors [26]. To determine whether a similar phenomenon takes place when expressing the Env protein of VISNA, we studied apoptosis in VV-*env*-MV infected cells. By different criteria of apoptosis measurements, like DNA and RNA degradation and presence of cytoplasmic DNA-histone complexes, we have concluded that expression of VISNA Env in BSC-40 cells infected with VV-*env*-MV does not trigger apoptosis (data not shown). This is a relevant observation if this or similar recombinant viruses expressing VISNA Env are to be used in future vaccine development.

Discussion

VISNA has been shown to infect and productively replicate *in vivo* in cells of the monocyte/macrophage lineage [19]. Differences among VISNA strains having different target organs (brain vs. lungs) and different *env* and LTR sequences have also been described with regard to the *in vitro* viral growth rate in sheep

choroid plexus cells [1]. The basis for this restricted cellular tropism and viral growth rate has not been elucidated. As yet, the cell receptor for VISNA has not been identified. Although binding of VISNA to MHC class II antigens has been reported [16], it appears that expression of MHC class II molecules is not sufficient to render non-permissive cells susceptible to VISNA infection. More recently, a role for a proteoglycan in VISNA cell binding and infection has been reported [3]. Early experiments in tissue culture using vesicular stomatitis virus (VSV) pseudotyping indicated that the VISNA receptor was present on cell lines derived from many non-ungulate species from a variety of tissue origins [2, 20, 29]. In this regard, using our recombinant VV-*env*-MV, Lyall et al. (2000) have shown that the receptor for VISNA is widely distributed, is more similar to that of amphotropic type C retroviruses and mapped to chromosomes 2 and 4 [28]. In retroviruses, the Env glycoprotein is responsible for the fusion mechanism that mediates the entry of the virus into the host cell after interaction with the cellular receptor [18], as well as for the fusogenic cytopathic effect observed in susceptible cells. Research on VISNA Env glycoprotein has been hampered by the lack of reagents to study such structure/function relationships. Although expression of the analogous CAEV Env protein (surface and transmembrane polypeptides) has been achieved in CAEV-free cells infected with CAEV-*env* recombinant VV [12, 27], previous attempts to express the Env protein using the entire VISNA *env* sequence in different systems have been unsuccessful, apparently due to toxicity caused by hydrophobic domains in the VISNA Env protein. In this investigation, a recombinant VV that contains the full DNA sequence of the VISNA *env* gene was generated, and it was used to demonstrate that the VISNA Env precursor gp150 is efficiently expressed in cells infected by this recombinant virus. By both metabolic labeling and Western blot analysis of VV-*env*-MV infected cells we could only distinguish the presence of the glycosylated precursor gp150 and higher molecular weight aggregates that appear to correspond to oligomers of gp150. We could not detect the presence of the surface gp135 and transmembrane gp46 cleavage products. This may be due to inefficient proteolytic cleavage of gp150 precursor in these cells, or alternatively, the anti-gp135 antibody was poorly immunoreactive, and may not detect gp135 when present in low abundance. We consider the latter possibility unlikely as the anti-gp135 is polyclonal, gives good reactivity in immunoblots and the same uncleaved gp150 product was observed when serum from VISNA infected sheep was used (see Fig. 4). A possibility existed that lack of Env processing was related to the expression of the protein in cells which are non permissive for VISNA replication. However, this seems not to be the case, since only the gp150 product was observed in VV-*env*-MV infected ovine fibroblasts. Since recombinants of VV are known to overproduce the recombinant product, the fact that we did not observe cleavage of gp150, neither in whole cell lysates nor in cell supernatants, strongly suggests that in VV-*env*-MV infected cells Env of VISNA is poorly or not at all processed. However, the Env protein appeared to be properly transported to the plasma membrane, since extensive syncytium formation was observed at late postinfection stages. Interestingly, despite the VISNA restricted cell tropism, syncytium formation

was found in cells from different origins infected with the recombinant VV, a phenomenon that is abolished when Env glycosylation is prevented. These results indicate that upon infection of these cell lines with the recombinant VV, the VISNA Env protein undergoes the necessary post-transcriptional modifications required to be transported to the cell membrane and to activate cell-to-cell fusion. While inhibition of chondroitin sulfate GAG chains addition to proteoglycans prevents entry of VISNA to cells [3], we have found that blocking proteoglycan synthesis enhances cell-to-cell fusion of Env from VISNA delivered by a VV recombinant. Possibly removal of GAG chains facilitates the exposure of the fusion domain of Env and their further interaction between cell membranes, triggering cell-to-cell fusion events. This fusion phenomenon is quite effective, and requires, if any, limited cleavage. Since during VISNA infection there is cleavage of gp150, our findings suggest that other factors (cellular or viral) are needed for gp150 cleavage during natural VISNA infection.

The Env glycosylation observed in VV-*env*-MV infected cells and the restricted cleavage may be advantageous for vaccine development based on this VV recombinant, since non neutralizing antibodies, which may increment the affinity of the virus for the receptor, appear to have low affinity for glycosylated proteins [22, 31]. Entry of VISNA into cells appears to require a receptor in addition to that which mediates fusion, as suggested by the observation that antibodies specific of VISNA Env that inhibit cell-to-cell fusion do not neutralize the virus [13, 14]. Thus, different regions of Env may interact with different receptors. On the other hand, host restriction may occur intracellularly at a later stage of the virus life cycle. The restricted productive replication of the closely related virus CAEV in ovine fibroblasts has been attributed to abnormal cleavage of the Env protein [10]. More recently, block of viral replication after entry of the virus into the cell has been shown to occur at virion assembly, as described in HIV-1 using a mouse model [30].

In conclusion, this investigation provides the first evidence for expression of the complete Env protein using the entire *env* sequence of VISNA in an VISNA-free system, revealed that Env is largely produced as a non cleaved product with a molecular mass of about 165 kDa, and demonstrates the ability of this protein to cause cell-to-cell fusion in cells of different origins. We showed that fusion-from-within required glycosylation and that removal of GAG chains increased the cell fusion phenomenon of this recombinant protein. The recombinant virus VV-*env*-MV described in this report, may represent a valuable tool to study further biological properties of the VISNA Env protein and could be instrumental in the development of a vaccine against VISNA.

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