Baculovirus expression and potential diagnostic application of the gp51 envelope glycoprotein of genetic mutants of the bovine leukaemia virus

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Abstract

Introduction: Field isolates of bovine leukaemia virus (BLV) show the presence of a few amino acid substitutions in major conformational G and H epitopes on surface glycoprotein gp51. Potentially, these substitutions can affect the 3D structure of these epitopes leading to their diminished immunoreactivity. The aim of this study was to express three gp51 glycoproteins carrying mutated epitopes as recombinant baculovirus proteins in insect cells to test their immunoreactivity with bovine sera.

Material and Methods: Env gene chimeras encoding mutated epitopes G and H in the env backbone of BLV FLK strain were constructed, cloned into pFastBac1 vector, and expressed in baculovirus. Results: The presence of recombinant gp51 protein in S9 insect cells was confirmed using monoclonal antibodies. ELISA tests were developed to check the immunoreactivity of recombinant protein with bovine sera. Conclusion: Recombinant gp51 proteins with altered G and H epitopes can be used for further studies to analyse the serological response of bovine sera towards BLV antigenic variants.

Keywords: bovine leukaemia virus, conformational epitopes, recombinant baculovirus protein.

Introduction

Bovine leukaemia virus (BLV) is a member of the Retroviridae family belonging to the Deltaretrovirus genus. BLV is recognised as the aetiological agent for enzootic bovine leukosis, a disease that results in significant economic losses in the cattle industry worldwide. BLV infection remains asymptomatic in the large majority of infected animals. Only approximately one third of BLV-infected cattle develop lymphocytosis, while in a minority of cases (approximately 5%), BLV infection leads to a lymphoma stage characterised by clonal expansion of B lymphocytes. Similarly to other complex retroviruses, the BLV genome contains the structural genes gag, pol, and env, regulatory genes tax, and rex, and accessory genes R3 and G4 (7).

Over the years, many studies on BLV genetic variability and molecular epidemiology have primarily and particularly focused on the part of the env gene encoding surface glycoprotein gp51 because of its biological functions. The extracellular gp51 protein plays a key role in the viral lifecycle, determining viral infectivity. It contains the receptor binding domain (RBD), which is indispensable for viral entry into host cells (8, 9). In addition, the localisation of gp51 glycoprotein at the surface of viral particles determines its role as a natural target of neutralising antibodies. Indeed, studies with different monoclonal antibodies revealed that the N-terminal portion of BLV gp51 contains three conformational epitopes, F, G, and H, which play an important role in eliciting neutralising antibodies and syncytium formation (2, 3, 14). This was confirmed by Forti et al. (6) who showed that conformational epitopes F, G, and H are located in the first 173 amino acids of the N-terminal portion of gp51 protein. Further studies with monoclonal antibodies revealed the existence among BLV isolates of different origins of antigenic variants characterised by different
expression of epitopes F, G, and H (4). In extension of this study, Mamoun et al. (11) identified the divergence at nucleotide and amino acid level among seven BLV strains and identified particular amino acids that contributed to the antigenic changes of conformational epitopes. This data was further extended over many studies with BLV isolates from different geographic origins, showing the presence of some amino acid substitutions, mainly within epitopes G and H (10, 12, 13, 15, 17). Potentially, these substitutions can affect the three-dimensional structure of epitopes or glycosylation of gp51 protein, leading to diminished immunoreactivity (11).

Our previous study identified the BLV isolates showing nucleotide variation in a part of the env gene encoding epitopes G and H in cattle infected with BLV (1). These mutations caused a significant amino acid variation leading to the following phenotypes: (F+G–H–GG–G–), (F+G–H–GG–G–), and (F+G–H–GG–G–) in the isolates from cattle #317, #306, and #18, respectively. Furthermore, for these animals, some discrepancy between the results of the PCR and the serological test was observed, which implies that inactivation of the epitope may diminish antigenicity and complicate diagnosis. In this paper, we constructed env gene chimeras encoding mutated epitopes G and H in the env backbone of the foetal lamb kidney (FLK) strain of BLV. Next, these chimeras were expressed as recombinant baculovirus proteins in insect cells and recombinant gp51 proteins were tested for their immunoreactivity with bovine sera with the aim of using them as potential diagnostic reagents.

Material and Methods

Construction of plasmids containing epitope-specific gp51 sequences. The gp51 gene was amplified from genomic DNA extracted from BLV-infected FLK cells with primers containing the PsiI restriction site and START (5'-GCTGGCAGGCTTCAATGC CT AAA-3') and STOP codon (UAA) recognition site (5'-CGACTTACTACGTCTGACC-3'). Then, the amplification product was cloned into pCRBlunt plasmid (Qiagen, USA), and an FLK-ATG-gp51-pCRBlunt plasmid was constructed. Plasmids carrying 286 bp fragments of the env gene encoding respective amino acids of epitopes G, H, GG, and G were constructed by nested PCR amplification of genomic DNA from peripheral blood mononuclear cells (PBMC) of cattle #317, #306, and #18 from the previous investigation (1) (Table 1), using two sets of primers (5'-CTCTCCGTCCCTTAGAAATC-3' and 5'-GGGGCTAATAGATCTTAGG-3'). After digestion with AvrII/BglII restriction enzymes, the fragments were ligated substituting the corresponding fragment of FLK-ATG-gp51/pCR Blunt plasmid, and as a result, recombinant plasmids containing epitope-specific sequences were created (FLK-ATG-gp51/317/pCRBlunt, FLK-ATG-gp51/306/pCRBlunt, and FLK-ATG-gp51/18/pCRBlunt). Plasmid FLK-ATG-gp51-pCRBlunt was used to generate wild-type gp51. Finally, DNA sequencing of the constructed plasmids was performed to confirm that no difference existed between the inserts and the original variants or between the env backbone of BLV FLK and the parental strain.

Construction of recombinant baculovirus vectors. The gp51 gene was recovered from plasmids carrying epitope-encoding sequences after digestion with PsiI and then subcloned into the baculovirus transfer vector pFastBac1 (Invitrogen, USA), using the PsiI restriction site. The recombinant baculovirus transfer constructs pFastBac1-317, pFastBac1-306, pFastBac1-18, and pFastBac1-FLK were used for site-specific transposition in E. coli (strain DH10Bac). Recombinant bacmids (baculovirus shuttle vector, large low-copy plasmid) containing the gp51 gene or gp51 variants were used to transfect insect cells (Sf9 cell) to produce recombinant baculoviruses. Recombinant proteins gp51/FLK or gp51/FLK/317, gp51/FLK/306, or gp51/FLK/18 variants were identified by the immunoperoxidase monolayer assay (IPMA) with monoclonal antibody to the D-D’ epitope of BLV (BLV2, Veterinary Medical Research and Development, USA) and 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich, Poland) as a substrate.

Expression and characterisation of recombinant gp51. The recombinant baculoviruses were used to infect Sf9 cells (5 x 10⁴) at low MOI and cell cultures were harvested 5–7 days post infection. The harvested cells and supernatants were separated by centrifugation at 13,000 rpm for 10 min. Culture supernatant, membrane, and cytoplasmic fractions were examined for recombinant gp51 protein by SDS/PAGE and western blotting using monoclonal antibody to the D-D’ epitope of BLV and peroxidase conjugated rabbit anti-mouse IgG (Sigma-Aldrich, Poland). Levels of expressed recombinant gp51 proteins were determined by enhanced chemiluminescence (ECL) using SuperSignal West Pico Chemiluminescent substrate (ThermoScientific, Poland).

ELISA with recombinant gp51 proteins. Four ELISA tests were performed with respective recombinant antigens. Immulux HB ELISA plates (Dynex Technologies, Germany) were coated with carbonate buffer-diluted culture medium at 1:10 collected after the fourth passage of Sf9 cells infected with recombinant baculoviruses gp51/FLK, gp51/FLK/317, gp51/FLK/306, and gp51/FLK/18. The
plates were incubated overnight at 4°C and then washed three times with PBST buffer with 20% horse serum (Sigma-Aldrich, Poland). The tested sera were diluted 1:100 in PBST buffer with 1% of horse serum and were incubated in duplicate for 1 h at 37°C. Then, the plates were washed five times, incubated with mouse IgG anti-bovine IgG labelled with peroxidase for 45 min at 37°C and washed again, and 100 μL of 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) solution was added to each well. After 15 min of incubation, the reaction was stopped and the optical density (OD) was measured at 405 nm. The tested sera included the positive control serum (P), which was prepared from five cattle naturally infected with BLV. All these animals’ samples were positive in PCR and in sequence analysis of amplified 444 bp fragments, confirming sequence conservation within epitopes F, G, and H with respect to the BLV FLK reference strain. The negative control serum (N) was prepared from five cattle seronegative in commercial ELISA and negative in PCR. Homologous sera were also tested from cattle designated #317, #306, and #18 in our preceding study, (1) which were naturally infected with BLV isolates.

Results

The sequence analysis of the cloned env gene revealed the presence of a single mutation C→T at position 514 resulting in one amino acid change (A→S) at position 172, as compared to the parental strain (BLV-FLK, GenBank accession no. M35242). However, this substitution was irrelevant for gp51 immunogenicity since it was located away from the region encoding epitopes and was frequently found in other isolates (17).

After transfection of Sf9 cells with recombinant bacmid DNAs, the monoclonal antibody to epitope D-D’ identified the gp51 backbone in cells infected by recombinant baculoviruses gp51/FLK, gp51/FLK/317, gp51/FLK/306, and gp51/FLK/18 but not in cells infected with wild-type baculovirus (WT) (Fig. 1). Expression of gp51 protein evaluated by SDS-PAGE in cell lysates (membrane fraction and cytoplasm) and supernatant showed for each construct a single protein band of molecular weight of 51-kDa, corresponding to the expected size of the Env protein (data not shown). In Western blotting developed by ECL, the expressed protein was reactive with a monoclonal antibody to epitope D-D’ of BLV (Fig. 2). The recombinant gp51 protein band was clearly visible in the supernatant fractions collected from cells infected with FLK and three variants, suggesting that the recombinant protein was released into the medium of the infected Sf9 cells. The protein band representing the membrane fraction was also visible in the form of a “smear” typical for the glycosylation process in endoplasmic reticulum. No band of the corresponding molecular weight was observed when Sf9 cells were infected with wild-type baculovirus.
Finally, an ELISA was performed to assess the reactivity of recombinant baculovirus proteins with bovine sera. Different dilutions of supernatant fractions and of control and homologous sera were used to optimise the results. The ELISA carried out with recombinant gp51/FLK antigen reacted effectively with P control serum since the resulting OD value was about 2.5 times higher than with N control serum (Table 2). In contrast, no cross reactivity was noted between recombinant gp51/FLK antigen and homologous sera.

Similarly to gp51/FLK, high binding capacity was observed for the recombinant gp51/FLK/306 antigens which reacted with respective homologous sera but not with P control serum. Interestingly, in ELISA, the G– and H–epitope-lacking antigen gp51/FLK/317, generated from Sf9 cells infected with recombinant baculovirus, interacted poorly with homologous serum since the OD value was comparable to that noted for negative serum N.

**Table 1.** Phenotypes and epitope nucleotide sequences of BLV wild type (FLK) and genetic variants used in this study

| Phenotype of epitope       | Epitope G | Epitope H | Epitope GG | Epitope G       |
|----------------------------|-----------|-----------|------------|-----------------|
| FLK (G+H+GG+G+)           | GCA (Ala) | TCC (Ser) | GCC (Ala)  | AAG (Lys)       |
| #317 (G–H+GG–G–)         | ACA (Thr) | TCC (Ser) | CCC (Pro)  | AGG (Arg)       |
| #306 (G+H+GG–G–)         | GCA (Ala) | TCC (Ser) | CCC (Pro)  | AGG (Arg)       |
| #18 (G–H+GG–G–)          | ACA (Thr) | TCC (Ser) | GCC (Ala)  | AAG (Lys)       |

**Table 2.** OD values of ELISA tests showing the reactivity of different recombinant antigens with serum samples

| Antigen       | Homologous sera | Positive control serum | Negative control serum |
|---------------|-----------------|------------------------|------------------------|
| gp51/FLK      | 0.389           | 0.611                  | 0.669                  |
| gp51/FLK/317  | 0.503           | 0.705                  | 0.597                  |
| gp51/FLK/306  | 0.320           | 0.610                  | 0.393                  |
| gp51/FLK/18   | nd              | 0.755                  | 0.543                  |

**Fig. 2.** Western blot-ECL analysis of recombinant baculovirus proteins. B – proteins in membrane fraction, C – proteins in cytoplasmic fraction, and P – proteins in culture medium. WT – cells transfected with wild type baculovirus, M – Amersham High Range Rainbow protein ladder (Sigma-Aldrich, Poland)
tests employing monoclonal conjugate directed to defined epitopes are used.

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