Expression of a ScFv-E2T fusion protein in CHO-K1 cells and alfalfa transgenic plants for the selective targeting to antigen presenting cells

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a pestivirus of the *Flaviviridae* family. It is an important cause of mortality, morbidity and economic loss of cattle with a worldwide distribution. Vaccination is an important component of BVDV prevention and control programs. Subunit vaccines provide the opportunity to develop safe vaccines. However, the challenge is to generate a protective immune response to a cost affordable for veterinary applications.

One alternative to solve this problem is to increase vaccine immunogenicity. A central event in the development of an adaptive immune response is the activation of CD4⁺ T cells by antigen presenting cells (APCs). APCs present antigenic peptides to CD4⁺ T cells on MHC class II molecules. In consequence, a way to obtain a more intense specific immune response is to increase the number of MHC-peptide complexes on the surface of APCs. This would be possible by targeting antigens to APCs, fusing them to specific antibodies against APCs' surface markers. In this work, we report the development of a fusion protein between a single chain antibody fragment (ScFv), recognizing a conserved region of MHC class II molecules, and a truncated form of the E2 glycoprotein (E2T) from BVDV, without its transmembrane domain. E2 is the most immunogenic protein of the virus and therefore is a good candidate for a subunit vaccine.

Another alternative is the use of transgenic plants as vectors for the expression of viral and bacterial antigens. Transgenic plants have been increasingly tested as an alternative methodology for the production of experimental vaccines, based on their economical growing facilities, scaling-up simplicity, low risk of pathogen contamination, no requirements for purification and possibility of oral administration. Feed fodder crops such as alfalfa are the best option for the development of animal vaccines using transgenic plants. However, a major drawback of using transgenic plants as protein factories is the low expression level of the recombinant antigens. The use of strong promoters for conducting transgene expression may permit to overcome this problem. Previous results obtained by our group employing transient expression systems in alfalfa (*Medicago sativa*), showed that Cassava Vein Mosaic Virus (CsVMV) promoter presents higher transcriptional activity than the standard 35S promoter. Here we report the generation of alfalfa transgenic plants expressing ScFv-E2T and E2T using the CsVMV promoter. Furthermore, both proteins were expressed transiently and stably in CHO-K1 cells and purified from cell culture supernatants as a convenient source of purified protein for functional studies.

RESULTS

Transient expression of ScFv-E2T and E2T in CHO-K1 cells. CHO-K1 cell monolayers were transfected with different plasmid concentrations. After 24 hs., cell lysates were evaluated by SDS-PAGE and Western blot for the presence of recombinant proteins. Incubation with a monoclonal antibody specific for E2 revealed the presence of the desired proteins (Figure 1).

Stable cell line generation. Transfected cell monolayers were subcultured in the presence of 700 µg/ml Geneticin (Invitrogen) for 21 days. Transfection with a plasmid containing the LACZ coding region was used for monitoring the progress of the selection process. After 14 days, groups of cells resistant to the antibiotics (blue-coloured) were evident, as revealed by beta-galactosidase activity using X-Gal as substrate (Figure 2). At day 14 and 21, cells were subcultured by limiting dilution in 96-well microplates in order to obtain stable and amplified recombinant clones. Positive clones were detected by Western blot for both constructs (red-circled, Figure 3). These clones were amplified and stored in liquid nitrogen.

Figure 1. Western blot analysis of cell lysates 24 hs. post-transfection

Figure 2. Cells transfected with LACZ coding region and incubated with Geneticin (G418). Visualization of beta-galactosidase activity by fixation and incubation with X-Gal (substrate). Cells expressing the transgene are blue.

Figure 3. Western blot analysis of supernatants from clones isolated by limiting dilution. Red-circled bands represent positive clones for each construct. NC: negative control. PC: positive control.

Characterization of recombinant ScFv-E2T and E2T expression in stable cell lines. Recombinant protein in cell culture supernatants was measured by ELISA. Clones with highest expression levels were chosen to carry out future experiments. Results indicated that product accumulates exponentially during the first 7-8 days and then reaches a plateau (Figure 4). Furthermore, addition of DMSO (2 %) and sodium butyrate (2 mM) to cell culture medium notably increased expression levels of recombinant proteins (4-fold increase). Product accumulation kinetics was similar using flasks and roller bottles, with even better yields in the latter case (data not shown).

Recombinant protein purification by IMAC. ScFv-E2T and E2T were purified by IMAC. Most proteins of cell culture supernatant remained unbound to the resin whereas recombinant proteins could be obtained in eluates with high purity (Figure 5). In the case of ScFv-E2T, some protein degradation is evident in spite of having used protease inhibitors. Proteins' identity was confirmed by Western blot. Recombinant proteins could be obtained with a purity of approximately 80 % and an average yield of the whole process of 40 %, being capable of purifying 134 µg of recombinant protein from 1L of cell culture supernatant (with an initial concentration of 334 µg/L) (data not shown).

Figure 4. ScFv-E2T and E2T expression in stable cell lines. Supernatants aliquots were taken for the first 8 days of culture and analyzed by ELISA. DMSO (2 %, red line) and sodium butyrate (2 mM, green line) were added to test their effect on transgene expression. Mean $\pm$ Standard Error (N = 4)

Figure 5. ScFv-E2T and E2T expression in stable cell lines. Supernatants aliquots were taken for the first 8 days of culture and analyzed by ELISA. DMSO (2 %, red line) and sodium butyrate (2 mM, green line) were added to test their effect on transgene expression.

Binding of ScFv-E2T and E2T to the surface of PBMCs. In order to test the ability of recombinant proteins to bind to the surface of APCs as the first step of antigen presentation, PBMCs from different species (bovine, pig, horse, goat, sheep, guinea pig and mice) were purified from heparinized blood with Ficoll-Paque Plus (Amersham), and used for surface labeling by flow cytometry. ScFv-E2T was capable of binding to the surface of PBMCs of all species except for mice, most presumably due to the monoclonal antibody from which these ScFv was derived was generated in mice (Figure 6). Furthermore, the targeted protein (ScFv-E2T) bound more intensively to PBMCs than the untargeted protein (E2T), demonstrating that targeting proteins to MHC class II molecules consist of an efficient strategy to augment antigen delivery to antigen presenting cells.

Figure 7. Surface labeling of PBMCs from different species with recombinant proteins. Results are expressed as the mean fluorescence intensity (MFI)

In vitro T cell proliferation by bovine APCs. To investigate if the improved antigen delivery to the surface of APCs mediated by targeting to MHC class II molecules resulted in a concomitant increase on the number of MHC-peptide complexes on the surface of APCs and ultimately in a higher T cell activation, an *in vitro* T cell proliferation assay was developed using bovine APCs (mainly macrophages). ScFv-E2T generated a higher T cell proliferation measured by ³[H]thymidine incorporation than E2T at all concentrations (Figure 8). Moreover, E2T seemed to be not capable of inducing a T cell proliferation of the magnitude and intensity as ScFv-E2T accomplished. ScFv-E2T required an approximately 10 times lower concentration in order to stimulate the same T cell proliferation induced by E2T. In consequence, ScFv-E2T seems to be capable of inducing T cell proliferation at even lower concentrations, whereas E2T could not.

Characterization of recombinant ScFv-E2T and E2T expression in alfalfa transgenic plants. Alfalfa transgenic plants were analyzed for the expression of E2T and ScFv-E2T fusion protein by ELISA. Expression levels in plants were very similar to those obtained in CHO-K1 cells (Figure 9), extracting approximately 1 µg of protein per gram of leaf (data not shown).

Figure 8. *In vitro* T cell proliferation generated by recombinant proteins and measured by ³[H]thymidine incorporation. Results are expressed as counts per minute (CPM). Mean $\pm$ Standard Error (N = 4)

Figure 9. Expression levels in alfalfa transgenic plants (grey bars) and comparison with E2T stable cell line supernatant (red bar). Mean $\pm$ Standard Error (N = 2)

CONCLUSIONS

- Recombinant proteins kept, in practical terms, the expected functional structure.
- Binding of recombinant proteins to the surface of PBMCs revealed that ScFv-E2T (targeted antigen) bound more intensively than E2T (untargeted antigen), demonstrating that targeting proteins to MHC class II molecules consist of an efficient strategy to increase antigen delivery to APCs.
- In an *in vitro* bovine model of T cell proliferation, ScFv-E2T generated a more potent specific T cell response than E2T. This means that antigen delivery to APCs through targeting to MHC class II molecules would result in a vaccine with higher immunogenicity than the conventional non-targeted vaccine.
- Expression levels in alfalfa transgenic plants were very similar to mammalian cells and estimated in 1 µg of recombinant protein per gram of leaf, providing evidence for the viability of using transgenic plants as system for subunit vaccine production.

MATERIALS AND METHODS

Single chain fragment variable (ScFv) construction. ScFv was developed from a mouse monoclonal antibody against pig MHC class II molecule (1F12).

Cloning of a truncated form of the E2 glycoprotein from BVDV (E2T). Total RNA from BVDV (NADL strain)-infected MDBK cells was isolated and reverse-transcribed by standard techniques. A truncated form of the E2 lacking its transmembrane domain (E2T) was amplified from the cDNA obtained and cloned in pGEM-T Easy vector (Promega). A modified version of E2T, lacking a start codon at the 5' end of the sequence, was amplified and cloned at the 3' end of ScFv in order to obtain ScFv-E2T.

Transfection and selection of stable CHO-K1 cell lines producing recombinant proteins. E2T and ScFv-E2T coding regions were subcloned in the mammalian expression vector pcDNA 3.1 (Invitrogen). CHO-K1 cells were transfected with expression vectors using LipofectAMINE 2000 (Invitrogen). Twenty four hours post-transfection cells were subcultured in the presence of 700 µg/mL of Geneticin (Invitrogen) for 21 days. At day 14 and 21, cells were subcultured by limiting dilution in 96-well plates in order to obtain stable and amplified recombinant clones. Positive clones were amplified and stored in liquid nitrogen.

Western blot. Proteins from cell culture supernatants, cell lysates or purified proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and incubated with a 1:1,000 dilution of a monoclonal antibody specific for E2. After extensive washing, bound antibodies were detected by chemiluminescence (Perkin Elmer) with horseradish peroxidase-conjugated anti-mouse IgG.

ELISA. U-bottom polystyrene microplates (Maxisorp, NUNC) were coated with an anti-E2 mouse monoclonal antibody (1:100 dilution). Samples were added at the corresponding dilution and incubated 1h at 37°C. As primary antibody, sera from rabbits immunized with E2T (1:2000) were used. Peroxidase-conjugated goat anti-rabbit IgG (1:250) were used as secondary antibody. The reaction was visualized with ABTS (Sigma) and absorbance was measured at 405 nm.

Protein purification by Immobilized Metal Affinity Chromatography (IMAC). Stable cell line supernatants were collected after 7-8 days of culture, centrifuged for 30 min. at 2,000 $\times$ g and used for protein purification by IMAC. PMSF and leupeptin were added as protease inhibitors. A 10 $\times$ Binding/Washing (BW) buffer (NaCl 3M, NaHPO₄ 0.5M) and 13 mM imidazole were added to cell cultures in order to increase ionic strength and reduce inespecific protein binding. Ni-NTA resin (Qiagen) was used at a relation of 2 mL supernatant. Supernatants were incubated overnight with shaking at 4°C. The resin was washed with BW buffer 1 $\times$ (containing 13 mM imidazole) until absorbance at 280 nm was negligible. Protein was eluted from the resin with BW buffer 1 $\times$ containing 200 mM imidazole. Eluates were washed with PBS and concentrated using Amicon-Ultra 4 centrifugal filter devices (Millipore). Purity and protein concentrations were determined by SDS-PAGE and Coomassie blue staining using a BSA standard curve. Purified proteins were stored at -70°C.

Binding of ScFv-E2T and E2T to the surface of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from heparinized blood using Ficoll-Paque Plus (Amersham). 5 $\times$ 10⁶ cells were used per assay and incubated with: 1) recombinant proteins (0.5 µg, 30 min. at 4°C); 2) anti-E2 monoclonal antibody (30 min. at 4°C); 3) FITC-labeled rat anti-mouse IgG (30 min. at 4°C). Analysis was performed using FACScan flow cytometer and CellQuest software (BD Biosciences).

In vitro T cell proliferation by bovine APCs. Isolated PBMCs, derived from seropositive bovine animals, were cultured for 24 hs. with RPMI 1640 medium supplemented with 10 % FCS. Non-adherent cells were used as a source of responder T cells. Adherent cells, used as APCs (mostly macrophages), were seeded at 5 $\times$ 10⁴ cells/well in 96-well microplates and incubated with different molar concentrations of recombinant proteins for 2 hs. at 37°C. APCs were washed and lymphocytes added (2.5 $\times$ 10⁵ cells/well). Cells were co-cultured for 4 days. On day 3, cells were pulsed for 18 hs. with ³[H]thymidine (1 µCi/well). Cells were harvested using a semiautomatic cell harvester (Skatron Instruments), and the amount of ³[H]thymidine incorporation was determined in a Wallac 1414 Winspectral beta scintillation counter (Perkin Elmer).

Production of alfalfa transgenic plants. ScFv-E2T and E2T coding regions were cloned in the plant expression vector pCAR, derived from pBI 121 (Clontech). Its main feature is the replacement of the 35S promoter by the stronger promoter CsVMV. Plants were generated by *Agrobacterium tumefaciens*-mediated transformation as described in [1].

REFERENCES

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