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RESEARCH ARTICLE

Membrane-spanning domain of bovine foamy virus transmembrane protein having cytotoxicity

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Abstract Foamy viruses (FVs) have broad cellular tropism infecting vertebrates from fish to human being, which indicates that Env protein has a high capability for membrane fusion. Conservative features in all FV transmembrane (TM) proteins include a region of hydrophobic domain called membrane-spanning domain (MSD), which contains several stretches of hydrophobic amino acids. To investigate whether these features were associated with the cytotoxicity effect of TM on *Escherichia coli*, a series of mutants were constructed and expressed in the *E. coli* BL21 (DE3) using pET-32a (+) as expressing vector. The results showed that only TM3 without MSD was expressed in *E. coli*, whereas the other two containing full or part of the MSD (TM1 and TM2) could not be expressed. Furthermore, the bacterial amount and living bacteria analysis revealed that the cytotoxicity of TM was dependent on its MSD, especially on the stretches of hydrophobic amino acids. Western blotting analysis showed that TM3 protein was purified with affinity purification.

Keywords bovine foamy virus (BFV), membrane-spanning domain (MSD), cytotoxicity

1 Introduction

Infection by retroviruses and other enveloped viruses is initiated by fusion of the virus with cell membranes, with the subsequent delivery of the viral genome into the host cell. Viral envelope glycoproteins play a critical role in this process, which tends to distribute itself in the lipid bilayer of the viruses as well as in the infected cells, and induce membrane fusion (Ji et al., 1999). Recent studies on protein structure show that these kinds of proteins, including the E1 protein of hepatitis C virus (HCV), the NSP4 protein of rotavirus, the gp41 and gp120 of HIV, have at least one hydrophobic transmembrane region and can form transmembrane channels (Ciccaglione et al., 1998; Chang et al., 1999; Browne et al., 2000). These features make them very difficult to be expressed in *Escherichia coli*.

Foamy viruses (FVs) belong to a distinct group of retroviruses. They are characterized by widespread and broad cellular tropism (Linial, 2000). By electron microscopy, they appear as spheres, are coated with phospholipid bilayer and their protuberant spikes are composed of surface unit (SU) peptide and transmembrane (TM) peptide. Both the N-terminal of SU and C-terminal of TM terminals have a hydrophobic region. The former functions as signal peptide and the latter functions as anchor to locate TM on the membrane (Herchenroder et al., 1999; Holzschu et al., 1998; Morozov et al., 1997; Pietschmann et al., 1999; Pietschmann et al., 2000). Here, we use bovine foamy virus (BFV) full length cDNA to construct expression vectors with deletion in different regions of TM and investigate the cytotoxicity of membrane spanning domain (MSD) in TM. This work provides elementary data for further study on FVs' package and infection.

2 Materials and methods

2.1 Virus, bacterial strain, plasmid and reagent

BFV3026 was isolated and stored in our laboratory. *E. coli* DH5α and BL21 (DE3), plasmid pET-32a (+) were stored. Enzymes and isopropyl-β-D-thiogalatoside (IPTG) were obtained from Takara (Dalian, China). Chelating Sepharose was obtained from Amersham Pharmacia. Primary antibody was antiserum extracted from a BFV3026-infected rabbit. Secondary antibody was goat anti-rabbit immunoglobulin-G horseradish peroxidase (IgG-HRP), obtained from Sino-American Biotechnology Company (shanghai, china).

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2.2 PCR primer

Several sets of primers were designed to amplify the different coding regions of TM and SU (Table 1). *Eco*R I and *Xho* I recognition sequences were introduced into forward and reverse primers, respectively. All primers were obtained from Sangon (Shanghai, China)

2.3 PCR

Template was BFV3026 protovirus. Reaction condition was: 94ºC 3 minutes, 46ºC 45 seconds, 72ºC 2 minutes, 1 cycle; 94ºC 1 minute, 50ºC 45 seconds, 72ºC 2 minutes, 30 cycles; 94ºC 3 minutes, 46ºC 45 seconds, 72ºC 6 minutes, 1 cycle.

2.4 Cloning and identification of PCR products

After agarose electrophoresis, PCR products were recovered from bands of proper molecular weight. Digested by *Eco*R I and *Xho* I, they were cloned into pET-32 a (+). The plasmid transformed *E. coli* DH5α, then extracted, digested and sequenced.

2.5 Expressing target gene in *E. coli*

E. coli BL21 (DE3) were transformed with pET-32a, pET-TM1, pET-TM2, pET-TM3. Single clones were inoculated into 3 mL Luria broth (LB) medium (contains 100μg/mL ampicillin), incubated overnight at 37ºC with shaking. Inoculated LB medium was at 1:100 ratio and incubated, and induced with 1 mmol/L IPTG for 2.5–3 hours when OD_{500} reached 0.7–1.0. Cells were collected, lysed and run on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

2.6 Growth test after induction

Inoculated LB medium with the cell carrying the recombinant reaction was incubated at 37ºC with shaking until OD_{500} reached 0.8. After induced for 1, 2 and 3 hours with 1 mmol/L IPTG, we measured OD and counted the number of colonies on plates (containing 100μg/mL ampicillin).

2.7 Purification of the target protein

Transformed cells were lysed with ultrasonic and loaded supernatant onto Ni^{2+} affinity column. Then we washed the column with binding, wash and elution buffers in turns, and collected bound protein.

2.8 Determination of antigenicity of expressed protein

After SDS-PAGE, purified protein was transferred to nitrocellulose membrane and ran Western blotting (Sambrook and Russell, 1989).

3 Results

3.1 Cloning target gene

Analysis by Predictprotein server on EMBL showed that TM protein had an MSD at C terminal from amino acid 39 to 12. Based on this result, three pairs of primers were designed to amplify full-length TM gene, TM gene with 60 bp deletion from C terminal of MSD and TM gene without MSD, respectively, named as TM1, TM2 and TM3. All three PCR products have the same size as predicted, which are 1.3 kb (TM1), 1.2 kb (TM2) and 1.1kb (TM3) (Fig. 1 and Fig. 2). After confirmation by sequencing, PCR products were cloned into pET-32a(+) and these constructs were named pET-TM1, pET-TM2, pET-TM3, respectively.

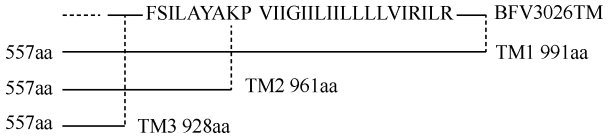

Fig. 1 Protein sequence of the membrane-spanning domain of BFV TM mature product and PCR products

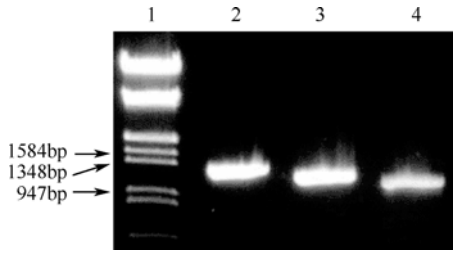

Fig. 2 Electrophoresis analysis of PCR products

3.2 Expression of target gene

E. coli BL21 cells with recombinant plasmids or pET32a were induced with IPTG for 2 hours. Only TM3 could be expressed with the correct molecular weight (63KD). The expressing protein accounted for 15% of the total protein of the bacteria (Fig. 3). Whereas, no detectable expression could be seen in pET-TM1 or pET-TM2 transforments.

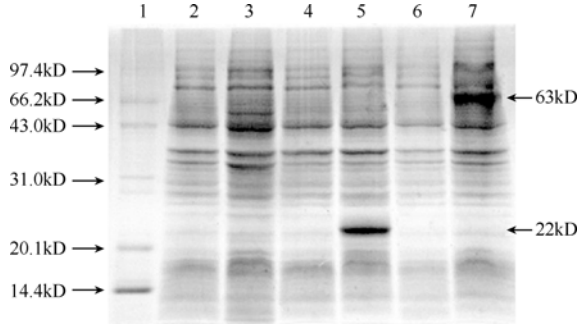

Fig. 3 SDS-PAGE Analysis of pET-TM3 expression product in *E.coli* BL21

3.3 Cytotoxicity of MSD

To determine the cytotoxicity of MSD, *E.coli* transformed with pET-TM1, pET-TM3 and pET-32a were induced with IPTG and cell growth was measured at different time points. The growth of pET-TM1 transformants kept on decreasing at all the time points, and the growth of pET-TM2 transformants increased slightly in the first hour then kept stable, while pET-32a transforments kept growing (Fig. 4). Furthermore, colonies counting were performed. After having induced with IPTG for 1 hour, the number of pET-TM1 transformants dropped from 10^8 cell/mL to 10^4 cell/mL, and pET-TM2 kept stable, whereas pET-32a transformants doubled its number (Fig. 5). These results suggested that MSD might be toxic to host cells and induce cell demise.

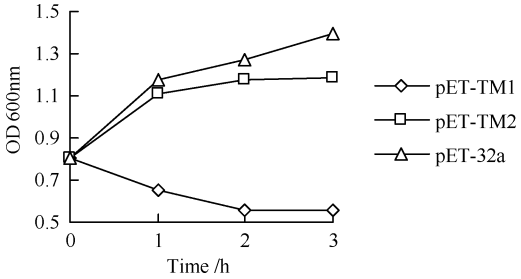

Fig. 4 Growth of *E. coli* BL21(DE3) transformed with expressing constructs and induced with IPTG

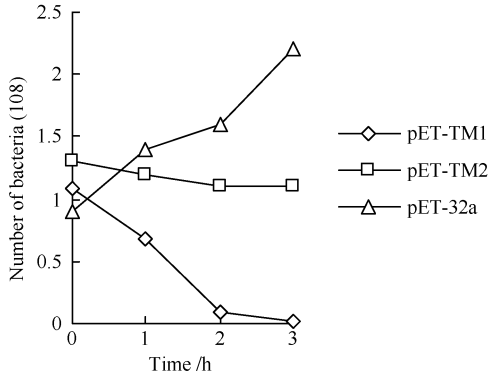

Fig. 5 Number of surviving bacteria transformed with expressing constructs and induced with IPTG

3.4 Protein purification and antigenic assay

TM3 protein was purified with Ni column and detected by SDS-PAGE and Western blot. The results showed that only one band could be detected and it could react well with BFV-infected rabbit serum, suggesting that purified TM3 had good immunoactivity (Fig. 6).

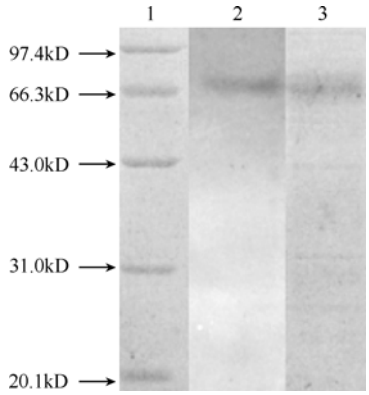

Fig. 6 Western blot of the purified TM3

4 Discussion

During viral entry, Env protein binds host cell membrane and mediates membrane fusion. In the course of virion maturity, Env protein takes part in packaging and releasing of viral particles (Ji et al., 1999). FV Env protein is encoded by the *env* gene. It is first translated as a full-length precursor protein and is then cleaved by cellular proteases into two mature subunits, SU and TM.

We have constructed a series of TM expression vectors with different deletion. After expression in *E.coli*, it was found that only the one without MSD can be expressed efficiently. Those with full or part MSD cannot be expressed. Furthermore, they can induce cell death. Sequence analyses show that TM MSD is composed of a number of tandem hydrophobic amino acid. These amino acids form a TM helix. It is supposed that the hydrophilic and hydrophobic amino acid can form polar and non-polar interfaces. The interfaces can bind the corresponding parts in cell membrane and perturb the membrane structure (Mangavel et al., 1998; Kim et al., 1999; Sheu and Lo, 1995; Lama and Carrasco, 1992). This can increase the membrane's permeability, inhibit cell growth and facilitate cell demise. Meanwhile, to decrease this effect, the host would eliminate plasmid or inhibit transcription or translation of toxic protein. In our study, transforments containing pET-TM1 or pET-TM2 are not stable, especially for the former (data not shown).

Not only BFV Env protein, but also HIV gp41, HCV E1 protein and rotavirus NSP4 protein are all toxic to host cells. This makes them difficult to be expressed in *E. coli*.

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