

Identification of Binding Proteins That Interact with BVDV E2 Protein in Bovine Trophoblast Cell

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Abstract—Bovine viral diarrhea virus (BVDV) can cause lifelong persistent infection. One reason for the phenomena is attributed to BVDV infection to placenta tissue. However the mechanisms that BVDV invades into placenta tissue remain unclear. To clarify the molecular mechanisms, we investigated the possible means that BVDV entered into bovine trophoblast cells (TPC). Yeast two-hybrid system was used to identify proteins extracted from TPC, which interact with BVDV envelope glycoprotein E2. A PGbkt7-E2 yeast expression vector and TPC cDNA library were constructed. Through two rounds of screening, three positive clones were identified. Sequencing analysis indicated that all the three positive clones encoded the same protein clathrin. Physical interaction between clathrin and BVDV E2 protein was further confirmed by coimmunoprecipitation experiments. This result suggested that the clathrin might play a critical role in the process of BVDV entry into placenta tissue and might be a novel antiviral target for preventing BVDV infection.

Keywords—Bovine viral diarrhea virus, clathrin, glycoprotein E2, yeast two-hybrid system.

I. INTRODUCTION

BOVINE viral diarrhea-mucosal disease (BVD-MD) is a complex disease with a variety of clinical manifestations induced by bovine viral diarrhea virus (BVDV). It is characterized by diarrhea, acute and chronic mucosal disease, immune tolerance and persistent infection, immunosuppression, congenital defects, abortion, stillbirths and fetal deformation [1]. BVD, like Acquired Immunodeficiency Syndrome (AIDS), is a special viral infection disease caused by vertical transmission through the placenta, which results in serious harms and great difficulties in the prevention and treatment [2]-[5]. Placental trophoblast cells (TPC), the first layer of placental barrier, are the unique fetal cells in the maternal-fetal interface directly contacting with mother's immune system and play an important role in intrauterine infection of various pathogens [5], [6]. However,

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the process of BVDV entry in TPC has not been investigated thus far. In this study, BVDV shz132 isolates and bovine TPC were employed to study and determine the role of binding proteins in the infection process by constructing the cDNA library of bovine TPC, screening the cell proteins that interact with BVDV shz132 E2 envelope protein using the yeast two-hybrid system and screening the receptor proteins associated with BVDV infection in the TPC. This study will provide in-depth understanding of the link between BVDV and embryo TPC, and reveal the molecular mechanism of their interaction.

II. MATERIALS AND METHODS

A. Cells and Strains

Bovine TPC, BVDV shz132 strains and pGM-T-E2 plasmid were stored in our laboratory. *Saccharomyces cerevisiae* AH109 and Y187 were purchased from Clontech Company.

B. Reagent

MatchmakerTM Library Construction & Screening Kits, BD CHROMA SPINTM+ TE-400 Columns, YEASTMAKERTM Yeast Transformation System 2, YPD medium and SD/-Leu, SD/-His, SD/-Ura nutrition selective medium were purchased from Clontech Company. TNT T7 Coupled Reticulocyte Lysate System, recombinant RNasin Ribonuclease Inhibitor, Transcend Non-Radioactive Translation Detection Systems and Nuclease Free Water were purchased from Promega Corporation. Whatman 3 MM paper was purchased from Whatman Company. TRizol reagent was purchased from Invitrogen Corporation. TIANprep yeast plasmid extraction kit, Easy Yeast Plasmid Isolation Kit, dNTPs, T4 DNA ligase, Taq DNA polymerase, agarose, DNA Marker and DNA extraction kit were purchased from Beijing Tiangen Biochemical Technology Co., Ltd. IPTG, X-β-gal, ethidium bromide and calcium chloride were purchased from Sigma. X-α-gal was purchased from ANASPEC Company.

C. Bovine TPC cDNA Library Construction and Quality Evaluation

The bovine TPC were isolated, purified and cultured for four generations [7]. RT-PCR and LD-PCR were performed using the total RNA as template according to the MatchmakerTM Library Construction & Screening Kits User manual. The homologous recombination plasmid was transformed into yeast. Transformation plates were randomly selected for colony counting. PCR amplification was performed using the library insertion sequence primers (upstream primers

5'-TTCCACCCAAGCATGGTATCAACGCAGAAGTGG -3', downstream primer 5'-TATCGATGATGCCAC CCTCTAGAGGCCGAGGCGGCCGACA-3'). The length distribution of the library plasmid DNA insertion fragment was analyzed.

D. Construction of Bait Plasmid pGBKT7-E2

E2 gene digested by EcoR I and Pst I in plasmid pGM-T-E2 was connected with carrier fragments of plasmid pGBKT7-lam (16 °C, 24 h). The positive plasmids identified were transfected to yeast cells Y187 and cultured in the synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade) to exclude its own activation.

E. Experiments with Yeast

The yeast Y187 colonies containing plasmid pGBKT7-E2 (greater than 2mm) were cultured in SD/-Trp/Kana medium at 30°C with shaking (40 r/min) for 24 h. After centrifugation, the cells were resuspended in 2 ml of $2 \times$ YPD/Kana culture medium. The cells (cell number $\geq 1 \times 10^9$ cells/mL) were cultured with 1 mL TPC library yeast AH109 in yeast liquid medium (45 ml) at 30°C with shaking (40 r/min) for 24 h. After centrifugation, the cells were resuspended in $0.5 \times$ YPD/Kana medium and then spread on 50 plates (diameter 15 cm) containing synthetic dropout nutrient medium at 30 °C for 6-10 d. The colonies were transferred to the plates containing synthetic dropout nutrient medium with X- α -Gal selective marker. The activity of the enzymes was assayed. The blue positive colonies were selected for PCR amplification and the library target gene fragments were sequenced and analyzed.

F. Immunoprecipitation

Immunoprecipitation was used to verify the interaction between the bait protein BVDV E2 and trophoblast capture proteins. The universal primers provided by the kit were used to PCR amplify the bait and capture plasmids. TNT T7 in vitro transcription and translation system were prepared. The protein interaction was analyzed by SDS-PAGE gel followed by detection with specific antibodies.

III. RESULTS

A. Construction, Purification and Quality Assessment of Bovine TPC cDNA Library

Quality of total RNA was checked using 1% formaldehyde denaturing agarose gel electrophoresis, which showed that 28S, 18S and 5.8S rRNA bands were clear and that 28S/18S rRNA ratio was approximately 2:1, indicating that the integrity of total RNA was good (Fig. 1). LD-PCR products were subject to 1.2% agarose gel electrophoresis, which showed that the library double strand cDNA presented zonal distribution. The fragment size range concentrated within 0.2-4 kb and was relatively dense near a region about 0.5-2 kb.

To assess the quality of cDNA library, ten plates were randomly selected for colony count. The average number of colonies in one plate was 650. The cDNA library contained 1.3×10^6 transformants, reaching the requirements for building

a database. PCR results showed that the size of cDNA insert fragment of this library was between 0.2 and 4 kb (Fig. 2).

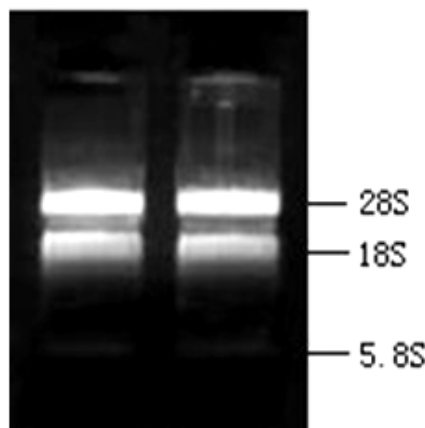


Fig. 1 Total RNA extracted from TGC

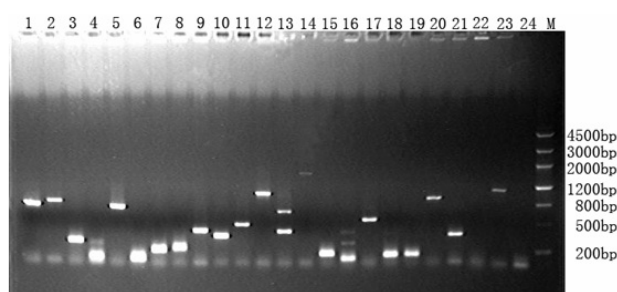


Fig. 2 Assessment of Bovine TPC cDNA library Lane 1-23, clones cDNA library; lane 24, negative control; lane M, molecular weight marker

B. Construction of Bait Plasmids and Yeast Screening

pGM-T-E2 and pGBKT7-LAM were subject to double digestion with restriction enzymes Pst I and EcoR I, respectively, and the expected target and vector fragment were obtained. After ligation and transformation, the positive recombinant plasmid pGBKT7-E2 (PCR fragment size 286bp) was identified and sequenced.

Next, we used the library primers to amplify the positive colonies, which could grow in both the synthetic dropout nutrient medium and X- α -Gal growth medium and turn blue. The gene fragments amplified were presented in Fig. 3.

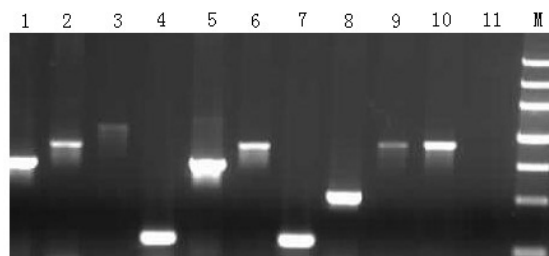


Fig. 3 PCR products for pGADT7-prey Lane 1-11, pGADT7-prey; lane M, molecular weight marker

Three colonies with consistent fragment size were selected for sequencing and one known gene clathrin was obtained.

The secondary structure of the protein was predicted using online website <http://www.expasy.org/cgi-bin/prosite/ScanView.cgi?scanfile=98781507182.scan.gz> and the function of different domains was speculated. The results showed that clathrin is a transmembrane protein with a heavy-chain linker and a putative tentative chaperonin binding epitope (NLILVVRG) structure located in the TPC surface.

C. Interaction of Bos Taurus Clathrin Heavy Chain (Hc) Capture Protein with E2 Protein

According to the instructions of Advantage 2 PCR Kit, the pGADT7-prey plasmid (*Bos taurus* clathrin) were PCR amplified and the corresponding DNA fragments with c-Myc antigen tag were obtained. The capture plasmids containing Clathrin gene and the E2 gene bait plasmids were subject to co-immunoprecipitation. The results showed that there was interaction between the two plasmids.

IV. DISCUSSION

E2 protein is the major glycoprotein of BVDV. Its N-terminal and membrane surface contain two epitopes. One is conservative within species and the other determines the antigen specificity of different strains [8], [9]. Therefore, N terminal is the main part that determines BVDV antigen, combines with BVDV antibody, mediates immune response and recognition of host cell [10]. In the present study, N-terminal of E2 protein was selected as the main antigen bait in species-conserved region. A strict control experiment in the yeast two hybrid system was performed to minimize false positive results by identifying individual activation report gene of the bait and target proteins. At the same time, a variety of screenings, such as nutrient deficiencies, antibiotic resistance, blue-white screening, were employed to greatly improve the specificity of protein interaction of the bait and capture proteins. In addition, the sensitivity of the strain and vector copy number was validated to avoid very low expression levels of the reporter gene resulting in undetectable results [11]. Based on this experiment, the immunoprecipitation technology was performed to verify the result. In the study of protein interaction using co-immunoprecipitation, the endogenous target protein was used as a bait to isolate protein complex [12], [13]. Therefore, not only can immunoprecipitation detect the interaction of natural complexes or all proteins in cell lysates with the target protein, but also exclude false positive.

Viruses penetrate target cells by two ways: 1) the receptor-mediated endocytosis and 2) the fusion of viral envelope and endocytosis membrane induced by acidic conditions at cell surface [17]-[21]. Clathrin-mediated endocytosis exists in many enveloped viruses such as Sindbis virus [22], Vesicular Stomatitis virus [23], Hantaan virus [24], and JC virus [25], Canine parvovirus [26], as well as Foot-and-mouth-disease virus [27]. BVDV is a small positive-strand RNA with a capsule. To date, little is known about the mechanisms how the BVDV enters cells. The

combination of BVDV with target cells is mediated by the interactions of E0 and E2 envelope protein with glycosaminoglycans and membrane proteins, respectively [14]-[16]. We screened and verified the interacts of *Bos taurus* clathrin (Hc) heavy chain with the BVDV E2 protein. Clathrin coat contains clathrin-substrate complex, which connects clathrin and receptors at small vesicles [28]. This endocytosis occurs at special areas in plasma membrane, which is known as clathrin-coated pits. Clathrin has no specificity and the clathrin-mediated endocytosis is modulated by specific adaptins [29]. Under the action of dyneins, the pits are broken at the neck away from plasma membrane and form clathrin-coated vesicles. Subsequently, under the action of Ca²⁺, clathrin separates from coated vesicles and returns to plasma membrane for recycle [30]-[32]. After leaving clathrin, the bare transitional vesicle fuses with early endosome and is further subject to intracellular digestion. Thus, we speculated that in the course of BVDV infection, after binding to cell membrane receptors, BVDV envelope protein concentrates in caveolin at cell membrane. Ligand-receptor complex is recognized and selected by adaptins of the caveolin, and then connected with clathrin by the heavy chain binding protein of "three-protein complex", resulting in endocytosis completion. If clathrin heavy chain binding protein is inhibited, the ligand-receptor complex proteins can not connect with the clathrin, which is required for the subsequent process of the cell internalization, leading to inability of virus to infect normal host cells.

Previous experimental results showed that in the process of BVDV infection of sheep embryos and lamb through the placenta, there was no difference in tissues, target organs and the distribution of target cells infected by virus, and that the viruses show strong tropism to epithelial cells, glial cells and lymphocytes [32]. The interaction of BVDV envelope protein E2 and the clathrin has no tissue-specificity, and the specificity of endocytosis mediated by clathrin is modulated by adaptins. Therefore, in the course of BVDV infection, clathrin-mediated endocytosis occurs in different tissues and organs in the process of vertical transmission.

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