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Identification and function analysis of the host cell protein that interacted with Orf virus Bcl-2-like protein ORFV125

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ABSTRACT

Orf virus (ORFV) causes contagious ecthyma, a non-systemic skin disease in sheep and goat. Bioinformatics analysis showed that ORFV125 has Bcl-2-like homologous domain and 3D structurally, it is generally known that Bcl-2 protein is known to be a key protein to control cell apoptosis. Maybe ORFV125 act as a Bcl-2-like manner to control cell apoptosis, but its exact function isn't very clear. So in this study, we use yeast two-hybrid system to identity the putative host cell protein interacting partners of ORFV125, and meanwhile using the data obtained from the Gene Ontology, Uniprot, and Kyoto Encyclopedia of Genes and Genomes databases to analysis the functions and pathways associated with them. Finally, five host proteins were shown to be interacted with ORFV125, including cytochrome b (cytb) gene, GUCY2C, BIRC5, GTF3C6 and SERBP1, we also found that BIRC5 has complex biological functions, can inhibit apoptosis, promote cell transformation and are involved in mitosis, and the interaction network of BIRC5 and ORFV125 were constructed. These findings provide a foundation to better understand the biology of the interactions between ORFV125 and the host proteins with which it directly interacts with and resultant downstream events.

1. Introduction

Poxviruses are masters of subverting cellular antiviral responses by encoding genes, which mimic, counteract or interact with various cellular proteins, and thus, can influence the course of infection, pathogenicity, and host tropism. Orf virus (ORFV) represents the type species of the genus *Parapoxvirus* (PPV) of Poxviridae, and causes contagious ecthyma (Buttner and Rziha, 2002). Sequencing of the genome of ORFV revealed 132 predicted open reading frames (ORF) resembling in organization and structure of other poxviral genomes (Delhon et al., 2004; McGuire et al., 2012; Mercer et al., 2006). The variable terminal regions of the genome comprises of genes dispensable for virus growth, some of them are unique for ORFV and encode factors important for virulence or pathogenesis of ORFV and are modulating the antiviral immune response of the host, including proteins that mimic the anti-apoptotic Bcl-2 like proteins ORFV125. Dana aligned 3 anti-apoptotic Bcl-2 family members (mouse Bcl-xL, human Mcl-1 and human Bcl-w) with the ORFV125 sequence incorporated, ORFV125 is predicted to have a Bcl-2-like structure (Hinds et al., 2007). The modeled ORFV125 structure resembled the Bcl-w protein more closely than Bcl-xL and Mcl-1, possessing 7 a-helices in similar positions and orientation as 7 of the 8 a-helices in Bcl-w (Petros et al., 2004).

Viral Bcl-2 homologs have been found in large DNA viruses such as herpesviruses, adenoviruses and poxviruses (Cuconati and White, 2002; Galluzzi et al., 2008). There is only one recognized Bcl-2 homolog in the poxvirus family; FWPV039 of *Fowlpox virus* (Afonso et al., 2000). However, a number of anti-apoptotic proteins have been identified which do not show substantial sequence similarities to Bcl-2 proteins, but function in a Bcl-2-like manner to inhibit apoptosis. These include the F1L and N1L proteins of *Vaccinia virus* (*Orthopoxvirus*), and the M11L protein of *Myxoma virus* (*Leporipoxvirus*) (Aoyagi et al., 2007; Cooray et al., 2007). Dana recently reported that ORFV, a member of the *Parapoxvirus* genus, encodes a unique anti-apoptotic protein, which shows little evidence of sequence similarities to other poxvirus protein (Westphal et al., 2007). Study also has identified an orf virus gene (*ORFV125*) whose presence was linked with the inhibition of apoptosis. The 173-amino-acid predicted protein had no clear homologs in public databases other than those encoded by other parapoxviruses. However, ORFV125 possessed a distinctive C-terminal domain which was necessary and sufficient to direct the protein to the mitochondria. Research has proved that ORFV125 alone could fully inhibit UV-induced DNA fragmentation, caspase activation, and cytC release and that its mitochondrial localization was required for its anti-apoptotic function.

So far we just known something about that ORFV125 has Bcl-2 manner to adjust apoptosis, to help better understand the detailed roles of

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ORFV125 in ORFV infection and pathogenesis, we performed yeast two-hybrid (Y2H) screening of the sheep testicular cells proteins that physically interact with ORFV125. By doing so, we identified five such sheep proteins, further characterization of the interactions between ORFV125 and these proteins will contribute to understanding of the function of ORFV125 during infection and pathogenesis.

2. Materials and Methods

2.1. Vector Construction

Sheep testicular cells (STCs) were preserved in our laboratory. Extracted ORF virus DNA were served as the template for PCR amplification of ORFV125, in conjunction with the following primers that had been designed using DNAStar software (DNAStar, Madison, WI): forward primer 5′-GAATTCATGGCAAACAGAGACGA-3′ (the *Eco*RI restriction site is underlined) and reverse primer 5′-GGATCCTTATGTGCGCCGCAA-3′ (the *Bam*HI restriction site is underlined). The PCR program was: 94 °C for 4 min; then 35 cycles at 94 °C for 50 s, 57 °C for 1 min, 72 °C for 2 min; 72 °C for 6 min. PCR product was excised from an agarose gel after electrophoresis and purified using the reagents of a DNA Purification Kit (Promega, Madison, WI). Restriction enzyme digestion and clone were employed to rapidly legate ORFV125 and pGBKT7 vector for the preparation of the bait plasmid pGBKT7-ORFV125.

2.2. Assessment of Autonomous Activation and Toxicity of pGBKT7-ORFV125

Competent Y2H Gold yeast cells were transformed with pGBKT7-ORFV125 as described in the *Yeast Protocols Handbook* (Clontech, Mountain View, CA), another yeast sample transformed with an empty pGBKT7 was served as the control. The competent transformed cells were individually cultured on tryptophan defective synthesis agar plates (SD/-Trp). Single colonies were screened by colony PCR (14) using T7-specific (5′-TAATACGACTCACTATA GGG-3′) and AD-specific (5′-TAAGAGTCACTTTAA AATTTGTATC-3′) as the primers. pGBKT7-ORFV125 transformed Y2H-Gold yeast cells were also plated onto tryptophan defective synthesis agar plates (SD/-Trp), Y2H Gold yeast cells transformed with pGBKT7-53 (Clontech, Mountain View, CA) and pGADT7-T (Clontech, Mountain View, CA) were plated onto tryptophan and leucine defective synthesis agar plates containing X-α-Gal and Aureobasidin A (SD/-Leu/-Trp/X-α-Gal/AbA) to provide a positive control. All plates were incubated at 30 °C for 3–5 days to observe the colony colors, sizes and numbers.

2.3. Construction of a the Y2H STCs cDNA Library

Total cell RNA was extracted from STCs with Trizol reagent (Invitrogen, USA), and then isolate mRNA using Oligotex mRNA Mini Kit (QIAGEN, Germany). For cDNA synthesis, the ends of the cDNAs were modified with the SMART III Oligo (5′-AAGCAGTGGTATCA ACGCAGAGTGGCCATTATGGCCGGG-3′) and CDS III Primer (5′-ATTCTAGAGGCCGAGGC GGCCGACATG-d(T)-3′) sequences, which are homologous to sequences in the prey vector pGADT-Rec (*Sma*I-linearized). The SMART III Oligonucleotide, which has an oligo(G) sequence at its 3′ end, base-pairs with the deoxycytidine stretch, creating an extended template. CDS III Primer hybridizes to the 3′ end of poly A+ RNA. In the majority of syntheses, the resulting ss cDNA contains the complete 5′ end of the mRNA as well as the sequence complementary to the SMART III Oligo, which then serves as a universal priming site (SMART anchor) in the subsequent amplification by long-distance PCR. Small DNA fragments were removed from the ds DNA preparation by passage through a CHROMA SPIN TE-400 column (Clontech, Mountain View, CA). Competent Y187 yeast cells were transformed with 20 μL of the ds cDNA and 6 μL of pGADT7-Rec (0.5 μg/μL) accordingly to the library-scale transformation protocol described in Yeastmaker Yeast Transformation System 2 User Manual (Clontech, Mountain View, CA).

After spreading 100 μL of 1/10 and 1/100 dilutions onto individual SD/-Leu agar plates (100-mm diameter), the plates were incubated at 30 °C for 3–4 days. Then the number of independent clones in the library were determined as an indication of library complexity. The remaining cells were spread onto SD/-Leu agar plates (150-mm diameter; 150 μL per plate), then incubated at 30 °C for 3–4 days until colonies appeared. The transformants were then harvested, pooled, mixed with YPDA liquid medium containing 25% (v/v) glycerol, divided into 1 mL aliquots, stored at −80 °C, and used within a short period of time. The cell density is about 8×10^7/mL.

2.4. Yeast Two-Hybrid Screening

Yeast colonies that had been transformed with pGBKT7-ORFV125 were individually cultured in 50 mL of SD/-Trp liquid medium shaking at 250 rpm, 30 °C until the OD600 of each culture was 0.8. The cultures were then centrifuged at 1000*g* for 5 min. The isolated pellets were suspended in 5 mL of SD/-Trp liquid medium and then used immediately.

For mating, 1 mL of the STC cDNA library was washed twice with 1 m L of 2 × YPDA liquid medium and mixed with bait sample (5 mL) in a sterile 2 L flask, and then added 45 mL of 2 × YPDA liquid medium containing 50 μg/mL kanamycin. Next incubated the sample at 30 °C for 20–24 h, with slow speed shaking (40 rpm). After centrifugation (1000*g* for 10 min) of the cells, the 2 L flask was rinsed twice with 50 mL of 0.5 × YPDA, (50 μg/mL kanamycin liquid medium). The rinses were combined and used to suspend the cell pellet, which was centrifuged again to pellet the cells (1000*g*, 10 min), which were then suspended in 10 mL of 0.5 × YPDA, 50 μg/mL kanamycin liquid medium, and about 50 aliquots (each 200 μL) of the yeast cells were plated on SD/-Leu/-Trp/X-α-Gal/Aureobasidin A(DDO/X-α-Gal/AbA) agar plates agar plates (150-mm diameter). After incubation at 30 °C for 3–5 days, blue colonies that appeared on the DDO/X-alpha-Gal/AbA medium. Given that murine p53 and the large T-antigen are known to interact in a Y2H assay, whereas Lambda does not interact with murine p53 protein, co-transformation with pGBKT7-p53 and pGADT7-T served as the positive control, and co-transformation with pGBKT7-Lambda and pGADT7-T antigen served as the negative control. All QDO/X-α-Gal/AbA positive interactions were assessed to identify duplicates and to verify that the interactions were genuine.

2.5. Rescue of the Prey Plasmids and Confirmation of the Positive Interactions

Blue colonies were selected and cultured in QDO liquid medium, the isolated yeast plasmids were individually transformed into *Escherichia coli* DH5 alpha competent cells. Plasmid DNA was then isolated from each culture using the AxyPrep Plasmid Miniprep Kit reagents (Axygen, Tewksbury, MA). To verify that ORFV125 and a host protein could really interact, the bait and prey plasmids were co-transformed into fresh Y2H yeast. Clones that appeared as blue colonies on QDO/X-α-Gal/AbA agar were considered a positive result, and using sequence analysis to verify the prey inserts. BLAST searches were performed to identify the corresponding proteins (http://www.ncbi.nlm.nih.gov/genbank/).

2.6. Function and Pathway Analysis

To further understand the biological functions of the host proteins identified as interacting with ORFV125, we retrieved the Gene Ontology (GO) (http://www.geneontology.org/) and Uniprot database (http://www.uniprot.org/) annotations. To identify the pathways in which the host proteins function, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) was searched.