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Short communication

Equine arteritis virus gP5 protein induces apoptosis in cultured insect cells

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ABSTRACT

Equine Arteritis Virus (EAV) has been shown to induce apoptosis *in vitro* but the induction of this mechanism has not been previously associated with any viral gene product. In this work, we found a cytotoxicity effect of the EAV gP5 protein on baculovirus-insect cells and a low yield of protein recovery. Besides, different morphological features by electron transmission microscopy, DNA fragmentation in agarose gel, TUNEL analysis and caspase 3 activity were found. All these findings indicate that the EAV gP5 protein induces apoptosis in insect cells.

1. Short communication

Equine Viral Arteritis (EVA) is a respiratory and reproductive disease of horses caused by Equine Arteritis Virus (EAV). EAV was first isolated in Ohio, USA, in 1953 (Doll et al., 1957a, 1957b) and has been classified as a member of the order *Nidovirales*, family *Arteriviridae*, and grouped together with lactate dehydrogenase-elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), and simian hemorrhagic fever virus (SHFV) (Cavanagh, 1997; Snijder and Spaan, 2001).

EAV is a small enveloped virus with a 12.7-kb positive-sense single-stranded RNA genome which includes nine functional open reading frames (ORFs) (Snijder and Meulenberg, 1998). ORFs 1a and 1b encode two replicase polyproteins (Snijder and Spaan, 2001) and the remaining seven ORFs (2a, 2b, and 3–7) encode the structural proteins of EAV. These structural proteins include four membrane glycoproteins (GP2 (25 kDa), GP3 (36–42 kDa), GP4 (28 kDa), and GP5 (30–44 kDa), encoded by ORFs 2b, 3, 4, and 5, respectively), two unglycosylated membrane proteins (E (8 kDa) and M (17 kDa), encoded by ORFs 2a and 6), and the phosphorylated nucleocapsid protein N (14 kDa), encoded by ORF 7 (Snijder et al., 1999; Wieringa et al., 2002).

In our laboratory, we have been studying the expression of EAV gP5 proteins as immunogen using baculovirus-insect cell system. The EAV gP5 gene was amplified using cDNA from the EAV strain LP02/C (GenBank reported partial ORF: DQ435441.1) (Echeverría et al., 2007). A pairs of primers with a restriction site sequence were then designed to allow the subsequent cloning into the pFastBacHT-B vector. The sense and antisense primers designed were: pFastgP5*BamH*I:5′-GGGGATCCGGCTCAACGATGTTATCT-3′ (nucleotides 11137–11154) and pFastgP5*Hind*III:5′-GGAAGCTTATGAATCTATGGCTCCCA-3′ (nucleotides 11902–11919). The underlined nucleotides denote the restriction enzyme sites added to the primers.

EAV gP5 PCR product was digested with the corresponding restriction enzyme and cloned into the pFastBacHT-B vector previously digested with the same restriction enzyme to generate the pFastBac-gP5 recombinant vector. This construction was confirmed by sequencing, using pFastBac Forward/Reverse Sequencing Primers.

Competent *Escherichia coli* DH10Bac cells, containing bacmid (baculovirus shuttle vector plasmid) and helper plasmid, were used to transform with pFastBac-gP5 and generate the recombinant bacmid (Bac-gP5) according to the Bac-to-Bac Baculovirus Expression System Manual (Invitrogen). The recombinant Bac-gP5 was

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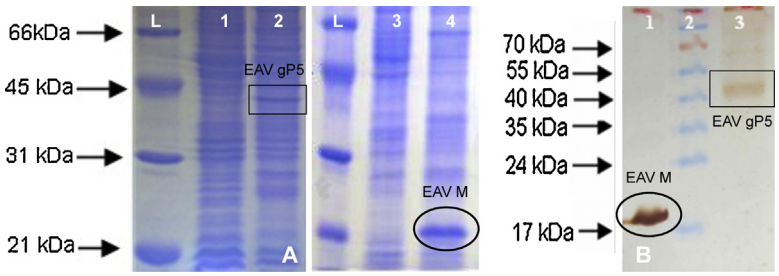

Fig. 1. [A] 12% SDS-PAGE. Total protein from High Five cells lysates transfected with recombinant baculoviruses. (L) Low molecular weight marker; (1, 3) non-infected High Five cells; (2, 4) Bac-gP5 and Bac-M infected High Five cells, respectively. [B] Western blot revealed with 1:3000 anti-histidine antibody. (1) Bac-M-infected High Five cells; (2) page ruler marker; (3) Bac-gP5-infected High Five cells.

transfected into a *Spodoptera frugiperda* cell line (*Sf*9) in serum-free medium using Cellfectin reagent (Gibco BRL).

Three days after transfection, the culture supernatant containing recombinant viruses (Bac-gP5) was harvested and subjected to standard plaque purification methods (Brown and Faulkner, 1977; King et al., 2007). Expression of recombinant gP5 protein was determined in High Five cells by SDS-PAGE and Western Blot with Anti-Histidine Antibody (GE Healthcare) (Fig. 1).

In our attempt to recover gP5 protein from Bac-gP5 recombinant baculovirus, we had problems of very low yields of protein expression as shown in SDS-PAGE and an unusual cytopathic effect observed in cultured insect cells as compared with recombinant baculoviruses carrying the EAV M (Bac-M). Infected High Five cells with Bac-gP5 did not show the rounded refringent cells characteristic of successfully infection. We found no previous reports of problems in the expression of EAV gP5 protein in a baculovirus system (Hedges et al., 1998) or other expression systems used to express this protein (MacLachlan et al., 1998; Weiland et al., 2000). Nevertheless, we found that previous reports have shown that gP5 protein from Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is associated with a strong cytotoxicity in cultured cells. This phenomenon has been associated with the induction of apoptosis (Suárez et al., 1996) by PRRSV gP5 protein. Also, other authors have reported that the first 119 amino acids, especially amino acids 90–119, constitute a fundamental region capable of inducing this mechanism (Fernández et al., 2002).

Apoptosis is one of the mechanisms by which nucleated eukaryotic cells die (Elmore, 2007) and is the innate mechanism by which organisms eliminate unwanted cells. Apoptosis is characterized by chromatin condensation, plasma-membrane blebbing, cell shrinkage, and DNA fragmentation into membrane-enclosed vesicles or apoptotic bodies (Häcker, 2000) and is triggered by a variety of stimuli such as UV radiation, chemicals and infectious agents.

Several viruses have been shown to be associated with induction of apoptosis (Del Puerto et al., 2011; Sur et al., 2000; Tran et al., 2013; Xu et al., 2012). Archambault and St-Laurent (2000) found that EAV induces this mechanism *in vitro* but they could not identify the virus genes responsible for its induction.

As mentioned above, apoptotic cells exhibit characteristic morphological features. Consequently, Bac-gP5-infected High Five cell monolayers were prepared for examination by transmission electron microscope. Bac-gP5-infected cells showed condensation of chromatin, nuclear fragmentation into apoptotic bodies and plasma membrane blebbing, observed also in sorbitol-induced apoptosis cells. Non-infected and Bac-M-infected cells did not show any of these features (Fig. 2). These morphological features were used as a first evidence of apoptosis in Bac-gP5 infected culture.

The incidence of nucleosome fragmentation by activation of intracellular endonucleases is associated with morphological changes in cells undergoing apoptosis (Rogalinska, 2002). Consequently, DNA was extracted from Bac-gP5 and Bac-M-infected cultures and from non-infected-cultured Hive Five cells using a

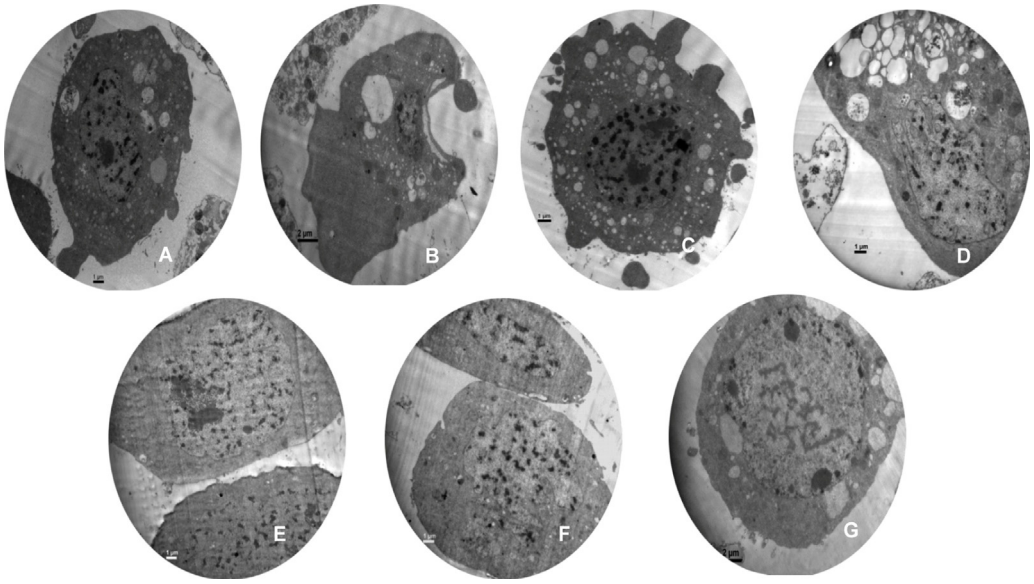

Fig. 2. Transmission electron micrographs taken from different infected and non-infected High Five cells at 72 h post-infection. Representative fields are shown. (A–C) Bac-gP5-infected High Five cells and (D) sorbitol-induced apoptosis High Five cells show morphological hallmarks of apoptosis included condensation of chromatin, the formation of apoptotic bodies and plasma membrane blebbing. (E and F) Non-infected cells and (G) Bac-M infected cells do not show distinguishable signs of apoptosis.