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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 citotoxicity 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.

Keywords: Equine Arteritis Virus, gP5 protein, apoptosis, insect cells.

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, 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 Meulemberg, 1998). ORFs 1a and 1b encode two replicase polyproteins (Snijder, 2001) and the remaining seven ORFs (2a, 2b, and 3 to 7) encode the structural proteins of EAV. These structural proteins include four membrane glycoproteins (GP2 (25 kDa), GP3 (36 to 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 to 11154) and pFastgP5*Hind*III:5'-GGAAGCTTATGAATCTATGGCTCCCA-3' (nucleotides 11902 to 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 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 (1999) 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 DNA Purification Kit (Promega). The results were analyzed by agarose gel electrophoresis, using sorbitol as a positive control of DNA fragmentation. The gel showed DNA fragmentation in Bac-gP5 and in sorbitol positive control. In contrast, no evidence of DNA fragmentation was observed in non-infected and in High Five cells infected with wild-type baculovirus (Bac) and with Bac-M (Fig. 3). This observed evidence of cellular apoptosis is due to the EAV gP5 expression in insect cells but has no correlation with the expression of EAV M protein or with baculovirus backbone.

Further, we analyzed the fragmentation of DNA in situ by using DeadEnd™ Colorimetric TUNEL System (Promega) to complement the above results. Briefly, in this system, biotinylated nucleotides are incorporated at the 3´-OH DNA ends by

using the terminal deoxynucleotidyl transferase. Horseradish peroxidase-labeled streptavidin is then bound to these biotinylated nucleotides, which are detected using peroxidase and diaminobenzidine (DAB) and with methyl green as a counterstain after completion of the immunohistochemistry. Using this procedure, apoptotic nuclei are stained dark brown. As it showed in figure 4, Bac-gP5-infected cells and sorbitol-induced apoptosis cells showed evidence of precipitated brown staining after 48hs post-infection compatible with colorimetric detection of DNA fragmentation. These observations were also evidence in DNAse-treated cells used as positive control. No evidence of apoptotic nuclei were found in any of the other assays made.

These results provide evidence that morphological changes previously observed in transmission electron microscopy are due to the activation of endonucleases. These endonucleases are cysteine proteases called caspases that have a central function in the amplification of cell death signal and consequently to the induction of apoptosis mechanism (Kumar, 2007). Caspase-3 is required for some typical hallmarks of apoptosis as chromatin condensation and DNA fragmentation in all cell types examined (Porter and Jänicke, 1999).

An immunodetection of caspase-3 in cultured High Five cells was made using Purified Rabbit Anti-Active Caspase-3 (BD-Biosciences-Pharmingen). The figure 5 showed a band at a predicted molecular weight of ~ 20 kDa corresponding to the activate caspase-3 in Bac-gP5 infected cells and in sorbitol positive control.

We speculated that EAV gP5 protein could play a role in the induction of apoptosis, as its counterpart, gP5 protein from PSRRV (Suárez et al., 1996). The

138 expression of EAV gP5 protein in insect cells evidences a poor accumulation and
139 strong cytotoxicity in infected cells due to apoptotic death, as observed with
140 expression of PRSSV gP5 (Fernández et al., 2002).

141 Collectively, the results obtained in this study indicate that EAV gP5 protein
142 induces apoptosis in insect cells. This is the first report that associates the
143 induction of apoptosis by an EAV viral product. The first evidence of EAV apoptosis
144 induction was observed in *in vitro* cultured Vero cells (Archambault and St-Laurent,
145 2000). It has been shown that this induction is initiated by caspase-8 activation and
146 subsequent mitochondria-dependent caspase-9 activation (St Louis and
147 Archambault, 2007) and that this mechanism of induction is mediated through the
148 intrinsic signaling pathway (Cholleti et al., 2013). Nevertheless, none of these
149 studies identified any viral gene product that could be a key factor in the induction
150 of apoptosis by EAV.

151 It has been documented that insect cells induce apoptosis as an antiviral
152 defense because of their lack of an adaptive immune response (Clarke and Clem,
153 2003). Baculoviruses are known to inhibit apoptosis in host cells (Clem et al.,
154 1991), a fact that has been correlated with at least two different types of
155 antiapoptotic genes, *p35* and *iap*, which prevent this mechanism in insect cells
156 (Clem, 2001). Normally, insect cells infected by baculoviruses do not exhibit
157 evident cytopathic effect until three days post-infection (Clem et al., 1991) because
158 these baculovirus-encoded proteins block apoptosis in early stages of virus
159 infection (Lacount et al., 1997), thus prolonging virus replication. We have found
160 DNA fragmentation in cells infected with baculovirus harboring EAV gP5 after 24hs

of infection (data not shown) correlating this results with the expression of this protein.

Ours results evidence that EAV gP5 protein is sufficient to trigger an apoptotic response in insect cells, despite the presence of these antiapoptotic proteins. Cultured-infected insect cells with wild-type baculovirus and baculovirus expressing EAV M protein did not show evidence of apoptosis with any of the methods used in this study indicating that the observed effect were due to the expression of gP5 protein in High Five cells. However, the mechanism of action in this activation is not clear. We hypothesize that the expression of EAV gP5 protein could inhibit the action of any of the antiapoptotic baculovirus proteins or induce apoptosis by a distinct apoptotic signal cascade that is not inhibited by these baculovirus proteins in insect cells. This induction of apoptosis may contribute to the low expression level of EAV gP5 recovery. Similar results have been found in the expression of Hepatitis C Virus E protein in insect cells (Ciccaglione et al., 2003).

As mammalian and insect cells share similarities in their apoptotic pathways, encouraged by this results our future experiments will attempt to identify and characterize this mechanism of EAV gP5 apoptosis induction in mammalian cells.

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Figure1: [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.

Figure 2: Transmission electron micrographs taken from different infected and non-infected High Five cells at 72 h post-infection. Representative fields are shown. (A, B, 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, F) Non-infected cells and (G) Bac-M infected cells do not show distinguishable signs of apoptosis.

Figure 3: Agarose gel electrophoretic pattern of DNA extracted from different cultured cells taken at 48h post-infection: (L) 100bp DNA ladder ; (1) Sorbitol-induced apoptosis High Five cells; (2) Bac-gP5-High Five infected cells; (3) Bac-M-High Five infected cells; (4) Non-infected-High Five cells; (5) Wild-type baculovirus-High Five infected cells

Figure 4: Several apoptotic cells by using Colorimetric TUNEL System showing the distinct condensation of the nuclear chromatin. (A, B, C) Bac-gP5-infected High Five cells; (D) Sorbitol-induced apoptosis High Five cells; (E) High Five cells incubate with DNAse. No evidence of apoptosis in non-induced cells (F) and Bac-M-infected High Five cells (G). Photographs were taken with 40x magnification.

Figure 5: Western Blot analysis using Purified Rabbit Anti-Active Caspase-3 . (L) Page Ruler Marker ; (1) Bac-gP5-High Five infected cells; (2) Non-infected High Five cells; (3) Bac-M-High Five infected cells; (4) Wild-type baculovirus-infected cells; (5) Sorbitol-induced apoptosis cells. The highlight bands of ~20 kDa corresponding to the active caspase-3. The bands observable at ~25 kDa represent the light chains of Ig used for the immunoprecipitation.

Highlights

- A recombinant baculovirus expressing EAV gP5 was constructed.
- The expression of EAV gp5 protein showed cytotoxicity effect in insect cells.
- PRRSV gP5 protein was associated with a strong cytotoxicity in culture cells.
- Several observed features in insect cultures were correlated with the induction of apoptosis.
- We identify a viral gene product that could be a key-factor in apoptosis induction.

315 FIGURE 1

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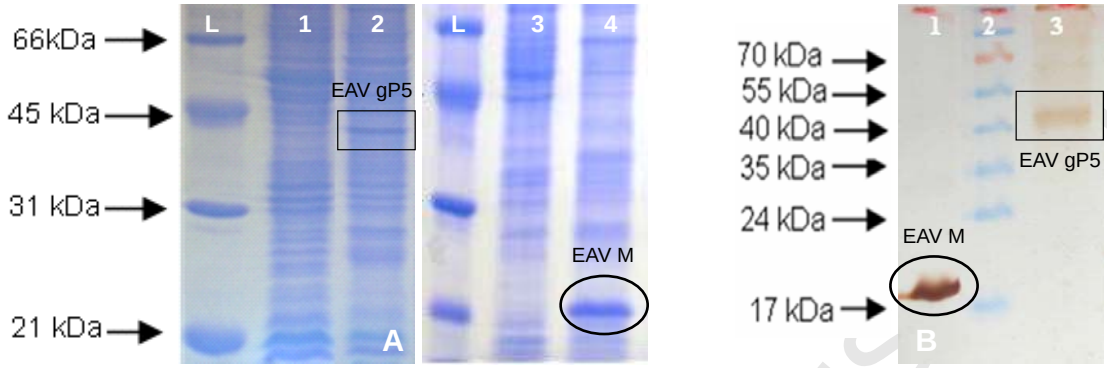

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319 FIGURE 2

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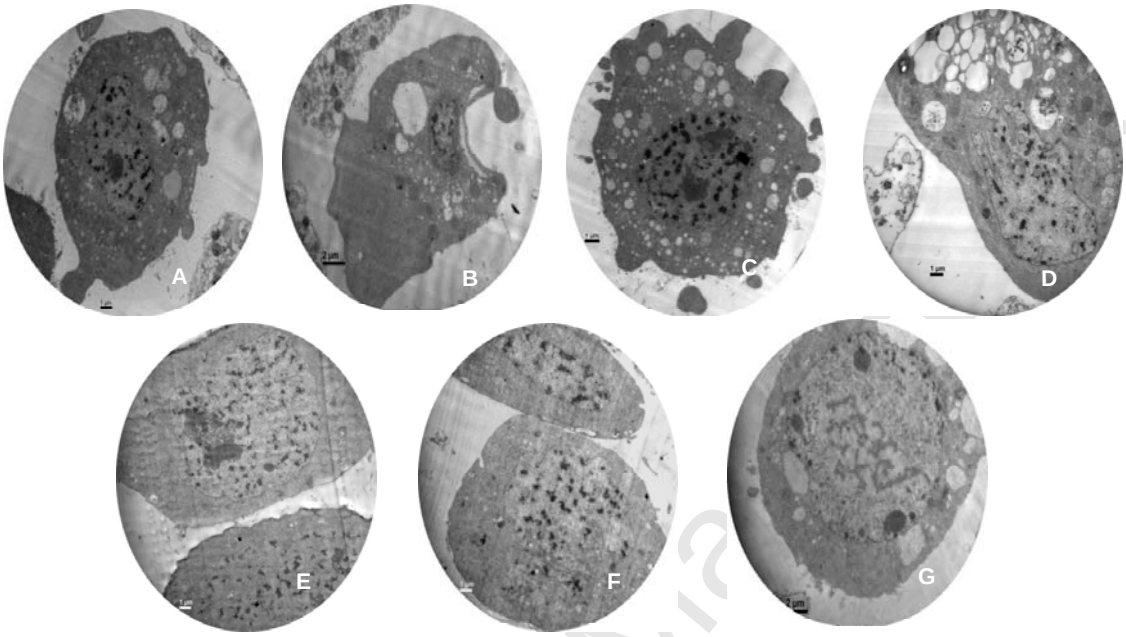

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322 FIGURE 3

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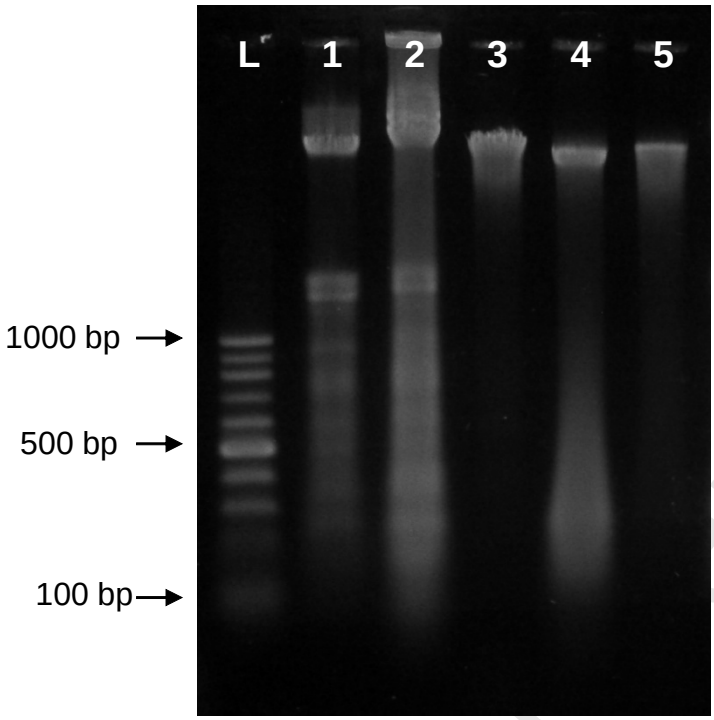

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328 FIGURE 4

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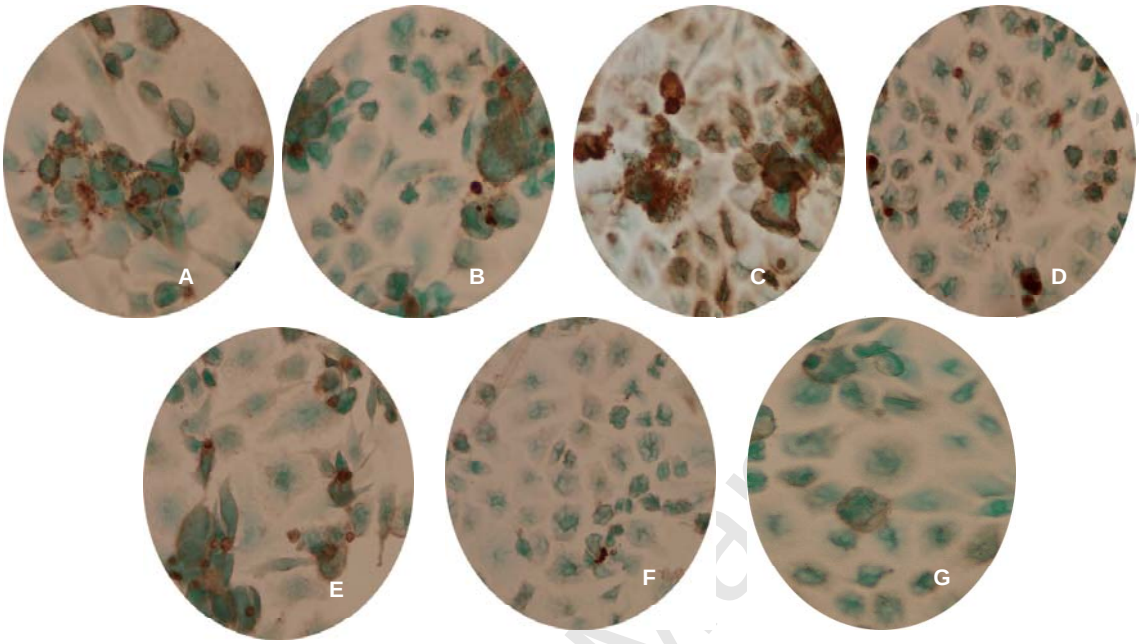

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337 FIGURE 5

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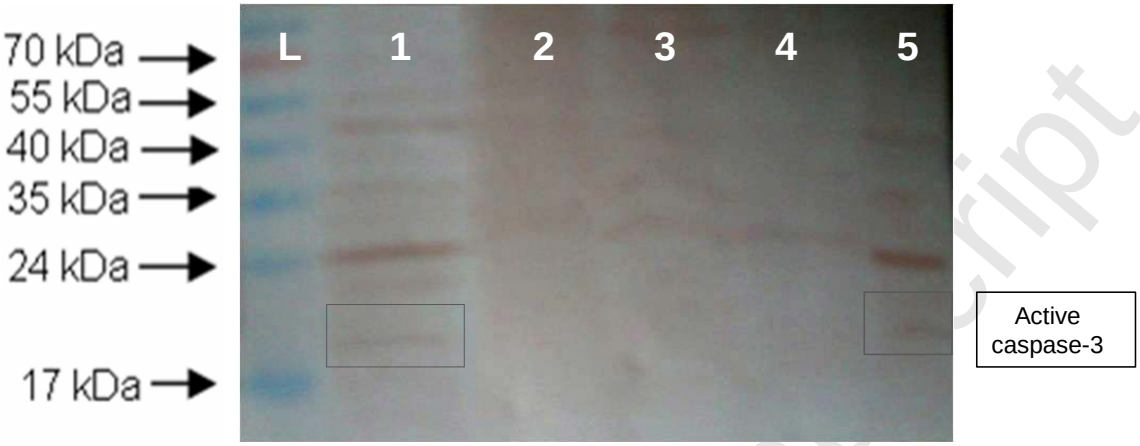

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