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A novel cell-based assay to measure activity of Venezuelan equine encephalitis virus nsP2 protease

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ABSTRACT

The encephalitic alphaviruses encode nsP2 protease (nsP2pro), which because of its vital role in virus replication, represents an attractive target for therapeutic intervention. To facilitate the discovery of nsP2 inhibitors we have developed a novel assay for quantitative measurement of nsP2pro activity in a cell-based format. The assay is based on a substrate fusion protein consisting of eGFP and Gaussia luciferase (Gluc) linked together by a small peptide containing a VEEV nsp2pro cleavage sequence. The expression of the substrate protein in cells along with recombinant nsP2pro results in cleavage of the substrate protein resulting in extracellular release of free Gluc. The Gluc activity in supernatants corresponds to intracellular nsP2pro-mediated substrate cleavage; thus, providing a simple and convenient way to quantify nsP2pro activity. Here, we demonstrate potential utility of the assay in identification of nsP2pro inhibitors, as well as in investigations related to molecular characterization of nsP2pro.

1. Introduction

The encephalitic alphaviruses include Venezuelan, Eastern, and Western equine encephalitis viruses (VEEV, EEEV, and WEEV, respectively) that belong to the genus *Alphavirus*, family *Togaviridae* (Schmaljohn and McClain, 1996). These viruses are widely distributed throughout the American subcontinent and are transmitted by hematophagous mosquitoes (Strauss and Strauss, 1994). They cause outbreaks of acute debilitating illness in humans and equid populations (Go et al., 2014). The human infection typically presents with flu-like symptoms, but may lead to encephalitis and even death. The mortality rate in human outbreaks ranges from $\leq$ 1% for VEEV, 3–7% for WEEV and 50–75% for EEEV. Even higher mortality rates have been recorded in equine outbreaks with 20–80% for VEEV, 70–90% for EEEV, and 3–50% for WEEV. Human infections of all three viruses have been reported in the United States (Zacks and Paessler, 2010). In addition to being a considerable public and veterinary health threat, VEEV and EEEV are also considered a significant bioterrorism threat due to their potential and/or history of development as a bioweapon (Reichert et al., 2009). Currently, no licensed therapy or vaccine is available against any of these viruses for human use.

Alphaviruses are small spherical enveloped viruses with a diameter of about 70 nm. The viral genome is packaged within an icosahedral capsid. The capsid is surrounded by a lipid envelope that is derived from host cell membrane during the process of virus budding. Embedded in the envelope are virally-encoded transmembrane glycoproteins that form spikes on viral surface (Rupp et al., 2015). The genome consists of a single-stranded, positive-sense linear RNA of approximately 11 kb in length (Strauss and Strauss, 1994). Like the cellular mRNAs, the alphavirus genomic RNA contains a 5′ cap and a 3′ poly-A tail, and is immediately translated upon release into the host cell cytoplasm. The 5′ two-third of the genomic RNA encodes nonstructural proteins called nsP1, 2, 3 and 4 that form the viral replicase complex. The remaining 3′ one-third of the genomic RNA encodes five structural proteins that include C, E1, E2, E3, and 6K that are transcribed from a subgenomic (26S) promoter (Strauss et al., 1984). Both structural and non-structural proteins are initially translated as precursor polyproteins that are later proteolytically processed to produce individual mature proteins (Jose et al., 2009; Strauss and Strauss, 1994).

The proteolytic processing of the nonstructural precursor polyprotein, designated as P1234, is carried out by the viral nsP2 protease (nsP2pro), which is located near the C-terminus of the full-length nsP2 protein (Hardy and Strauss, 1989). The enzyme contains a Cysteine-Histidine catalytic dyad and is included in the peptidase family C9 of clan CA (Rawlings and Barrett, 1993; Strauss et al., 1992). Structural analysis of VEEV nsP2pro indicated that the

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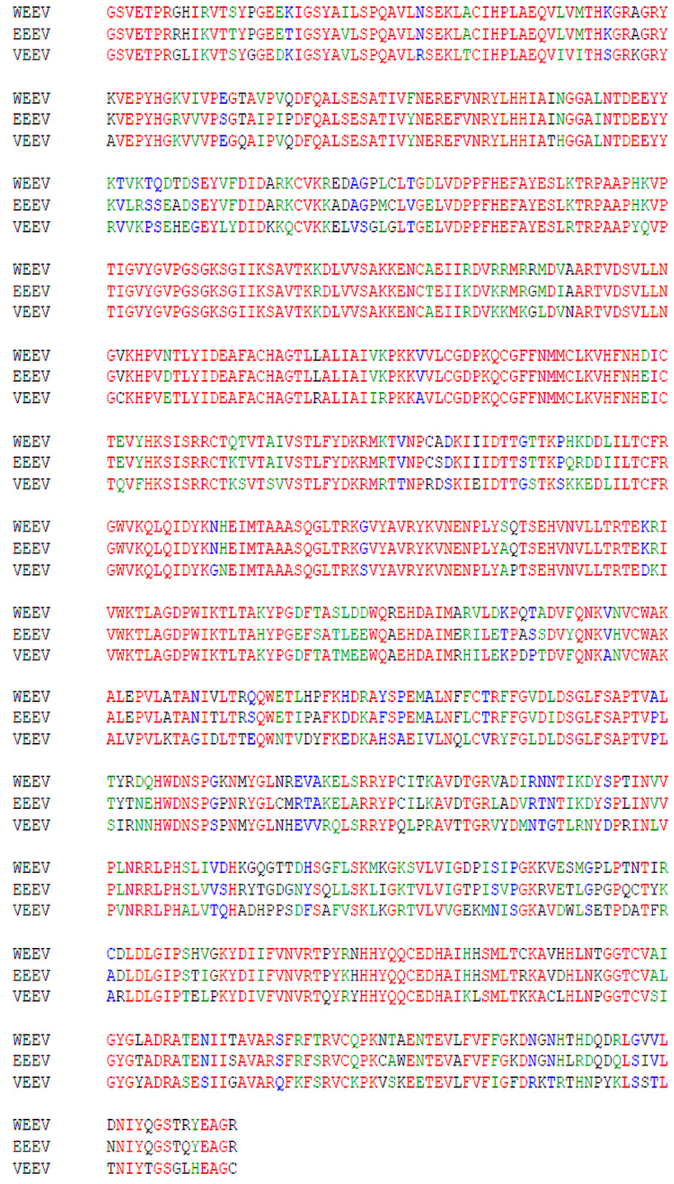

Fig. 1. Amino acid sequence alignment of WEEV, EEEV and VEEV nsP2pro. The sequences were obtained from UniProt data base and aligned using Clustal W program (Larkin et al., 2007). Sequences are from WEEV strain BFS1703 (UniProt identifier P13896); EEEV strain PE-3.0815 (UniProt identifier Q306W8) and VEEV strain Menaii (UnoProt identifier Q9WJC7). Red letters represent amino acids residues that are identical among the three sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

structure is organized into two discrete folded domains. The N-terminal subdomain contains the catalytic dyad, active site residues, and substrate binding sites S1–S3, and is organized in a protein fold that differs from any known cysteine protease or protein fold. The C-terminal subdomain has structural similarity to methyltransferases with no known enzymatic activity; however, it forms part of the active site and may be involved in substrate recognition and/or regulation of protease activity (Russo et al., 2006). The nsP2pro recognizes specific cleavage sites at the junction of nsP1/2, nsP2/3, and nsP3/4 within the precursor polyprotein (ten Dam et al., 1999). Since each individual non-structural protein is an essential component of the viral replicase complex, the correct processing and production of mature nonstructural proteins by nsP2pro is critical for the virus replication to proceed in an efficient and timely manner.

The vital role of VEEV nsP2pro in virus replication and its structural distinctness from host cell cysteine proteases make nsP2pro an attractive therapeutic target. Furthermore, the amino acid sequence that comprises nsP2pro exhibits high degree of sequence identity (67%) among VEEV, EEEV and WEEV (Fig. 1). This indicates that the enzyme is structurally similar among the three viruses. Similarly, the P4′-P4 amino acids at the nsP2pro cleavage sites are also conserved with the sequence at the nsP1/2 site being identical among VEEV, EEEV and WEEV, and only two amino acid differences at the nsp2/3 and nsP3/4 sites between VEEV and EEEV/WEEV (ten Dam et al., 1999). This suggests that a single nsP2pro inhibitor could potentially be active against all three encephalitic alphaviruses.

To exploit nsP2pro as a potential drug target, it is essential to have a specific and functional assay that could allow quantitative measurement of nsP2pro activity. Such an assay would immensely facilitate identification and evaluation of potential nsP2pro inhibitors. In this regard, a cell-based assay would be preferable since it would allow elimination of the membrane impermeable and/or cytotoxic inhibitors at the outset. To date, *in vitro* biochemical assays based on purified full-length nsp2 or nsp2pro have been reported for a few alphaviruses including VEEV (Pastorino et al., 2008; Strauss et al., 1992; Zhang et al., 2009); however, to our knowledge, no cell-based nsP2pro assay is currently available for any of the new-world encephalitic alphaviruses. Here we report the development of a simple and efficient assay for VEEV nsP2pro. The assay system is similar to that has been reported by others (Lee et al., 2003; Qu et al., 2014), and allows quantitative measurement of nsP2pro activity in a cell-based format. We present data demonstrating potential utility of the assay system in drug discovery efforts against VEEV, as well as in molecular studies related to VEEV nsP2pro.

2. Results

2.1. Design and validation of VEEV nsP2pro assay

To develop an assay that could be used to measure VEEV nsP2pro activity in cells, we needed a substrate whose cleavage by nsP2pro could easily be monitored and quantified. To this end, we designed a substrate protein that consisted of eGFP and Gaussia luciferase (Gluc) linked together by a small peptide containing the VEEV nsP2pro cleavage sequence based on the sequence found at the junction of VEEV nsP1/2 cleavage site (nsP1/2CS, Fig. 2(A)). Gluc is a small luciferase from the marine copepod *Gaussia princeps* that is naturally secreted when expressed in cells (Tannous et al., 2005); however, its fusion with a larger non-secretory protein such as eGFP reduces its secretion. It was expected that due to the presence of nsP1/2CS, the fusion protein would serve as a specific substrate for VEEV nsP2pro, and will be cleaved in VEEV-infected cells. This will lead to the separation of Gluc from eGFP and its subsequent secretion into the extracellular medium. Thus, the substrate cleavage could be monitored by measuring the Gluc activity in the cell culture supernatant and/or by Western blot analysis of cell lysates. In designing the substrate protein we chose the nsP1/2CS because the P4′-P4 residues at this particular site are completely conserved among VEEV, EEEV, and WEEV (ten Dam et al., 1999). We, therefore argued that the substrate protein could be used to measure the nsP2pro activity of any of these viruses without the need to change the nsP2pro target sequence.

To test the feasibility of the substrate protein in the assay system, we cloned the cDNA encoding the substrate fusion protein in a mammalian expression vector (see methods for details). The plasmid was then transfected into HEK293FT cells followed by