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Short communication

Identification of heat shock protein A9 as a Tembusu virus binding protein on DF-1 cells

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ABSTRACT

This study attempts to identify receptor elements for Tembusu virus (TMUV) on DF-1 cells. Using co-immunoprecipitation and virus overlay protein binding assays, we identified a TMUV-binding protein of approximately 70-kDa on DF-1 cell membranes. Mass spectroscopy identified the protein to be heat shock protein (HSP) A9, which was reconfirmed by an anti-HSPA9 antibody. Indirect immunofluorescence demonstrated a significant degree of colocalization between HSPA9 and TMUV on cell surface. Additionally, an antibody against HSPA9 could inhibit TMUV infection in DF-1 cells in a dose-dependent manner. These results might suggest that HSPA9 is a putative receptor for TMUV.

Tembusu virus (TMUV), a member of the Flavivirus genus in the Flaviviridae family, was first isolated from mosquitoes of the genus Cules in 1957 in Malaysia (Platt et al., 1975). In 2000, a subtype of TMUV, originally named Sitiawan virus, was isolated from sick broiler chickens with encephalitis and retarded growth (Kono et al., 2000), which confirms the pathogenicity of TMUV in domestic poultry for the first time. In April 2010, a novel TMUV emerged in eastern China and caused severe disease in ducks (Su et al., 2011; Tang et al., 2012; Yan et al., 2011). This virus spread rapidly around the major duck-producing regions of China in a few months and continued until the winter season, which affected more than 10 million ducks and resulted in a serious economic loss to duck industry (Li et al., 2012). At present, the novel TMUV has become one of the most economically important infectious agents of ducks in China, and also has been isolated from other domestic birds like geese, chickens, and pigeons, as well as wild species such as house sparrows (Chen et al., 2014; Dai et al., 2015; Han et al., 2013; Liu et al., 2012; Tang et al., 2013). As with other flaviviruses, TMUV is an enveloped virus and has a single-stranded and positive-sense RNA genome which contains a unique open reading frame (ORF). The ORF encodes a single polyprotein precursor which is cleaved by cellular and viral proteases into three structural proteins (C, PrM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4 B and NS5) (Mukhopadhyay et al., 2005). Viral attachment is the first step in virus infection (Smith and Helenius, 2004) and the interaction between virion and cellular receptor present on the surface of the host cell plays a crucial role in this step. A number of cell surface molecules have been identified as receptors in the cell entry process of other flaviviruses, such as glucose-regulated protein 78 on HepG2 cells for dengue virus serotype 2 (Jindadamrongwech et al., 2004), $\alpha_v\beta_3$ integrin on Vero cells for *West Nile virus* (Chu and Ng, 2004), and heat shock protein 70 on Neuro2a cells for Japanese encephalitis virus (Das et al., 2009). However, the actual cellular receptor molecule(s) that is involved in the attachment and entry of TMUV is unknown. This study therefore sought to isolate and characterize possible cell molecule(s) that acts as cellular receptor for TMUV.

To determine receptor proteins for TMUV, co-immunoprecipitation assay (Co-IP) was carried out as previously described (Lin et al., 2007) with some modifications. The membrane proteins from DF-1 cells were isolated using the Membrane and Cytosol Protein Extraction Kit (Beyotime, China) (Xu et al., 2013). The DF-1 cell membrane proteins were incubated with purified TMUV on a rocker at 4 °C for 6 h, followed by 4 h of incubation with a mouse specific anti-TMUV E protein polyclonal antibody. After that, protein A-agarose beads were added to the mixture and then incubated for overnight. The beads were washed three times and boiled in 2 × SDS loading buffer for 10 min. Co-immunoprecipitated complexes were then analyzed by SDS-PAGE

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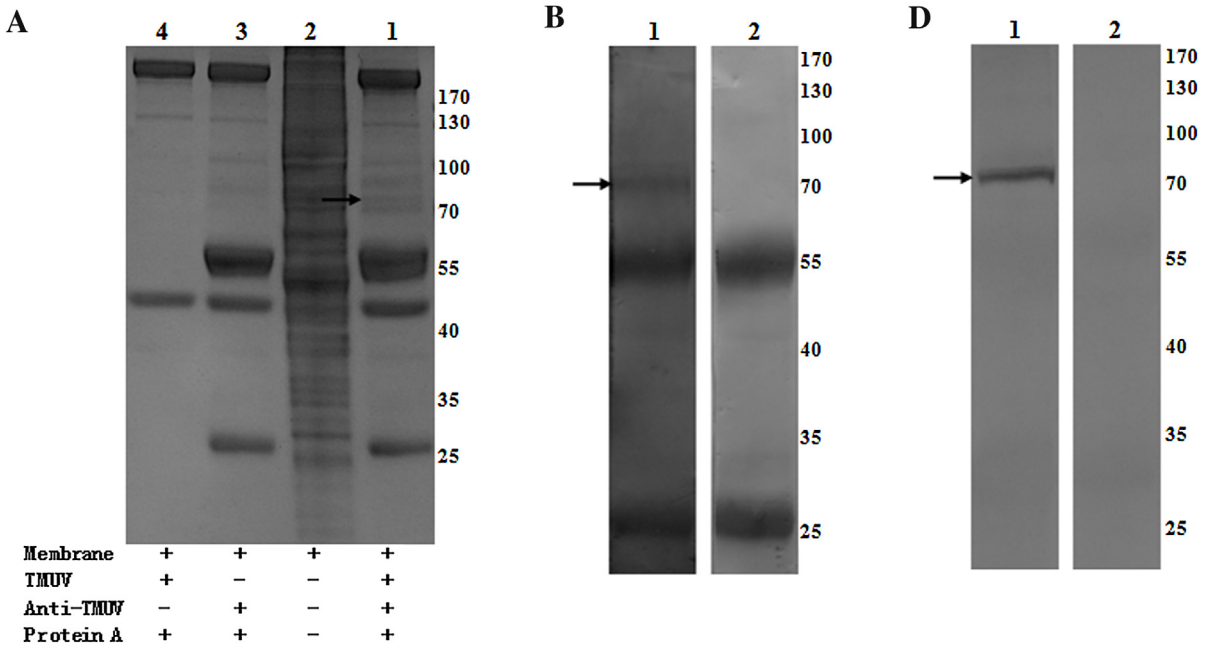

C

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631 tgenirqaat slqqaslklf emaykkmase ressgssgdq keekq
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Fig. 1. Identification of the TMUV-binding protein. (A) Detection of an approximate 70-kDa protein in DF-1 cell membranes by co-immunoprecipitated assy. The membrane proteins from DF-1 cells were immunoprecipitated with TMUV virions followed by TMUV E protein-specific antibody (lane 1), or with the TMUV E protein-specific antibody (lane 3) and TMUV virions (lane 4) alone. The co-immunoprecipitated complexes were then analyzed by SDS-PAGE gels stained with Coomassie blue. (B) VOPBA analysis of the co-immunoprecipitated complexes. The complexes were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated without (lane 2) or with 10^6 $TCID_{50}$ of TMUV (lane 1). Virus binding protein bands were detected via a pan specific anti-TMUV E protein monoclonal antibody. The approximate 70-kDa band is seen in lane 1 (black arrow). (C) Identification of the TMUV-binding band by mass spectrometry. The approximate 70-kDa protein band in the gel was excised and sent for commercial mass spectrometry fingerprint. The protein was identified as HSPA9. The peptide sequences identified by mass spectrometry are underlined. (D) Identification of the TMUV binding band by western blotting. The membranes containing the transferred co-immunoprecipitated complexes were incubated with a rabbit anti-HSPA9 antibody (lane 1) or normal rabbit IgG (lane 2), and then with an alkaline phosphatase-conjugated polyclonal goat anti-rabbit IgG antibody.

gels stained with Coomassie blue. As shown in Fig. 1A, a distinct band with approximate molecular mass 70-kDa was identified in the complexes co-immunoprecipitated with TMUV and anti-TMUV antibody (lane 1), while in the absence of TMUV (lane 3) or anti-TMUV antibody (lane 4), this band was not detected. Thus, we detected a TMUV-binding protein by co-immunoprecipitation assay. To validate this result, virus overlay protein binding assay (VOPBA) was carried out as described earlier (Das et al., 2009). The co-immunoprecipitated complexes were transferred to nitrocellulose membranes and incubated with TMUV, followed by sequential incubation with a pan specific anti-TMUV E protein monoclonal antibody (Zhao et al., 2015), and a goat anti-mouse IgG conjugated with alkaline phosphatase. As shown in Fig. 1B, TMUV recognized the protein of approximate molecular mass 70-kDa (lanes 1), while in the absence of TMUV, the anti-TMUV E protein monoclonal antibody was unable to detect the protein band (lane 2).

To identify the 70-kDa protein, the protein band in gel was excised and sent for commercial mass spectrometry fingerprint. This mass spectrum was compared with protein databases, and the protein was identified as heat shock protein (HSP) A9. Peptide sequences identified by mass spectrometry are shown in Fig. 1C. To further corroborate this identification, the co-immunoprecipitated complexes were transferred to nitrocellulose membranes and probed with a goat anti-HSPA9 antibody (Abcam, UK) which can react with chicken HSPA9, followed by the corresponding secondary antibody conjugated with alkaline phosphatase. As shown in Fig. 1D, the anti-HSPA9 antibody specifically recognized the approximate 70-kDa protein band. Collectively, these results indicated that the HSPA9 in the DF-1 cell membranes could interact with TMUV.

HSPA9, a member of the Hsp70 family of chaperones (also known as Mortalin/GRP75/mtHsp70), is associated multiple cellular functions ranging from stress response, intracellular trafficking, antigen processing, control of cell proliferation, differentiation, and tumorigenesis (Domanico et al., 1993; Dores-Silva et al., 2015; Sadekova et al., 1997; Wadhwa et al., 1993a, 2002). Although originally identified as a mitochondrial chaperone (Wadhwa et al., 1993a), HSPA9 is also detected in different subcellular