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The RNA binding of protein A from Wuhan nodavirus is mediated by mitochondrial membrane lipids

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ABSTRACT

RNA replication of positive-strand (+)RNA viruses requires the lipids present in intracellular membranes, the sites of which viral replicases associate with. However, the direct effects of membrane lipids on viral replicases are still poorly understood. Wuhan nodavirus (WhNV) protein A, which associates with mitochondrial membranes, is the sole replicase required for RNA replication. Here, we report that WhNV protein A binds to RNA1 in a cooperative manner. Moreover, mitochondrial membrane lipids (MMLs) stimulated the RNA binding activity and cooperativity of protein A, and such stimulations exhibited strong selectivity for distinct phospholipids. Interestingly, MMLs stimulated the RNA-binding cooperativity only at higher protein A concentrations. Further investigation showed that MMLs stimulate the RNA binding of protein A by promoting its self-interaction. Finally, manipulating MML metabolism affected the protein A-induced RNA1 recruitment in cells. Together, our findings reveal the direct effects of membrane lipids on the RNA binding activity of a nodaviral replicase.

Introduction

(+)RNA viruses replicate their genomic RNA in viral RNA replication complexes (vRCs) including viral replicase proteins, viral RNA, and host proteins, on specific rearranged intracellular membranes (Ahlquist, 2006; Ahlquist et al., 2003; Miller and Krijnse-Locker, 2008; Sasvari and Nagy, 2010). Different viruses form their vRCs on diverse intracellular organelle membranes, including the endoplasmic reticulum (Diaz et al., 2012; Lee et al., 2001; Mas and Beachy, 1999; Pedersen et al., 1999; Schlegel et al., 1996; Schmidt-Mende et al., 2001), Golgi apparatus (Schlegel et al., 1996), lysosomes (Froshauer et al., 1988; Kujala et al., 2001; Magliano et al., 1998; Schlegel et al., 1996), endosomes (Froshauer et al., 1988; Kujala et al., 2001), peroxisomes (Jonczyk et al., 2007; White and Nagy, 2004) and mitochondria (Miller and Ahlquist, 2002; Miller et al., 2001). Lipids are major components of intracellular membranes, as they control membrane fluidity and plasticity (Nohturfft and Zhang, 2009; van Meer et al., 2008), and membrane lipids facilitate the RNA replication of (+)RNA viruses (Miller and Krijnse-Locker, 2008). For instance, the complete activity of Semliki Forest virus (SFV) NSP1 protein, an mRNA capping enzyme, requires association with specific negative phospholipids (Ahola et al., 1999). Sphingomyelin has been found to activate the RNA-dependent RNA polymerase (RdRp) activity of Hepatitis C virus (HCV) genotype 1b (Weng et al., 2010). The precise functions of intracellular membranes, particularly membrane lipids, in the RNA replication of (+)RNA viruses have not been fully understood, but possibly include offering an optimal microenvironment for viral replicase enzymatic activities, facilitating the use of membrane-associated host cofactors and/or directly interacting with viral replicases to mediate their functions (Chukkapalli et al., 2012; Heaton and Randall, 2011).

Nodaviruses (family *Nodaviridae*) are (+)RNA viruses that contain a bipartite genome consisting of RNA1 (3.1 kb) and RNA2 (1.4 kb), which encodes protein A, the RdRP (Gallagher et al., 1983), and capsid precursor protein α (Schneemann et al., 1992), respectively. Moreover, a subgenomic RNA3 (sgRNA3), is synthesized during RNA1 replication and encodes protein B2, a suppressor of antiviral RNA interference (RNAi) (Li et al., 2002). Nodaviruses express a sole RNA replicase, protein A, which is responsible for both membrane association and viral RNA replication (Ball, 1995; Kopek et al., 2007; Miller and Ahlquist, 2002; Miller et al., 2001; Venter and Schneemann, 2008). This characteristic renders nodaviruses like Flock House virus (FHV) and WhNV simplified and ideal models for studying viral RNA replication.

In the case of FHV, the most extensively studied *Nodaviridae* member, its protein A is localized to outer mitochondrial membranes (Miller and Ahlquist, 2002; Miller et al., 2001). Previous studies of FHV indicated that membrane lipids mediate FHV RNA

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protein A function. The in vitro study showed that complete replication activity of FHV vRCs isolated from intracellular membrane require the addition of exogenous phospholipids (Wu et al., 1992; Wu and Kaesberg, 1991). FHV RNA replication in *Drosophila* cells can be blocked by inhibiting fatty acid synthesis (Kampmueller and Miller, 2005). Moreover, FHV protein A is a lipid-binding protein with particular affinity for specific anionic phospholipids, which may regulate the protein A-membrane interactions (Stapleford et al., 2009). However, although membrane lipids play an important role in nodaviral RNA replication, the detailed mechanisms by which membrane lipids regulate the activities of nodaviral protein A are not well understood.

As a virus closely related to FHV, WhNV has been well characterized and provides novel insights for nodaviral subgenomic RNA replication (Qiu et al., 2011), RNA silencing suppression (Qi et al., 2011; Qi et al., 2012) and initiation of RNA synthesis (Wang et al., 2013). Similar to FHV, WhNV protein A is associated with mitochondrial membranes (Qiu et al., 2013), and moreover, the membrane-association of WhNV protein A is closely linked with the recruitment and stabilization of viral genomic RNA1 on vRCs (Qiu et al., 2013). Furthermore, the in vitro data showed that WhNV protein A is self-interacted and the self-interaction of WhNV protein A is directly mediated by MMLs, suggesting the direct role of membrane lipids in WhNV protein A function (Qiu et al., 2014).

In this study, we focused on the direct effects of MMLs on the RNA-binding activity of WhNV protein A, because viral genomic RNA binding to viral replicases is important during many steps of the RNA replication, including the recruitment of the viral genomic RNA to the vRCs, recognition of replicating-elements, encapsidation of genomic RNA, assembly of the vRCs and activation of RdRp (Pathak et al., 2011; Pathak et al., 2012). To this end, we first examined whether WhNV protein A binds to RNA1 and RNA2 in vitro, and then explored the direct effects of MMLs on the RNA binding of protein A. We found that WhNV protein A binds to RNA1 in a cooperative manner, and the cooperativity could be stimulated by high protein A concentrations. MMLs stimulated the RNA-binding activity of protein A, and this stimulation exhibited strong selectivity for different phospholipids. Interestingly, MMLs stimulated the RNA-binding cooperativity only at higher but not at lower protein A concentrations. Further investigation showed that MMLs stimulate the RNA binding activity of protein A by promoting its self-interaction. Finally, we further confirmed that the MML composition changes by manipulating phospholipid metabolism affect protein A-induced RNA1 recruitment in cells.

Results

Characterization of the RNA probe used for determining the RNA binding of WhNV protein A

To determine whether WhNV protein A could bind to RNA directly, we first determined which RNA sequences can be used as the RNA probe. Previous study reported that nodaviruses contain the conversed sequences in the 5′-proximal region of RNA1 that exhibit a stem-loop structure and are responsible for RNA recruitment (Van Wynsberghe and Ahlquist, 2009). According to WhNV, its RNA1 nt 50–118 [$RNA1_{(50–118)}$] exhibited a similar secondary structure (Fig. 1B). To examine whether these sequences are responsible for WhNV RNA1 recruitment/stabilization in cells, we constructed two RNA1 derivates and examined their recruitment in the presence of WhNV replication-incompetent protein A_{GAA} (Fig. 1A). The plasmid pAC1E, in which an (enhanced green fluorescent protein) EGFP open reading frame (ORF) is inserted at the 3′ end of RNA1 sequence (Qiu et al., 2013), is a functional template for RNA1 replication (the transcribed and replicated products are labeled as "RNA1E"), but the ORF of protein A is closed by the mutation of the start codon (Qiu et al., 2013). WhNV Protein A_{GAA} is provided by the plasmid pA_{GAA}. The RdRp activity of Protein A_{GAA} is nulled by mutating the GDD replication site to GAA as previously described (Qiu et al., 2013; Wang et al., 2013). Protein A_{GAA} retained its RNA1 recruitment activity despite losing its RNA replicase activity (Qiu et al., 2013).

We determined the sequences required for RNA1 recruitment in Pr-E cells, which are derived from *Pieris rapae* larvae, the natural host of WhNV (Qiu et al., 2014). Thirty-six hrs after transfecting with the pA_{GAA} and the indicated plasmids, cells were collected and total RNA was analyzed by Northern blotting. As shown in Fig. 1C, the presence of protein A_{GAA} supported the recruitment of wild type (wt) RNA1E (compared lane 2 to lane 1; Fig. 1D). While destroying the stem-loop by either deleting (Fig. 1B, "Del") or mutating the stem structure (Fig. 1B, "Mut") of RNA1E resulted in the decrease in RNA1E recruitment (Fig. 1C, compared lane 4 or 6 to lane 2; Fig. 1D). These results indicate that WhNV RNA1 nt 50–118 is needed for RNA1 recruitment.

We sought to determine the sequences required for RNA2 recruitment in cells. The analysis of RNA2 sequence using RNA mfold showed that RNA2 nt 123–164 [$RNA2_{(123–164)}$] exhibits a stable stem-loop structure (Fig. 1E). We then examined whether this sequence is required for RNA2 recruitment by protein A in cells. Deletion of RNA2 nt 1–180 (Fig. 1F) resulted in a significant reduction in RNA2 recruitment in the presence of protein A_{GAA} (Fig. 1G, compared lane 4 to lane 2; Fig. 1H). On the other hand, RNA2 nt 1–180 was sufficient to support protein A-mediated RNA2 recruitment (Fig. 1G, compared lane 6 to lane 5; Fig. 1H). Furthermore, deleting the stem-loop region (Fig. 1E) resulted in a significant decrease in RNA2 recruitment (Fig. 1G, comparing lane 8 to lane 2; Fig. 1H). These results indicate that WhNV RNA2 nt 123–164 is required for RNA2 recruitment. Together, both $RNA1_{(50–118)}$ and $RNA2_{(123–164)}$ can be used as the RNA probes for the gel mobility shift experiments.

The characterization of the RNA1 binding behavior of protein A revealed its cooperative RNA binding

Maltose-binding protein (MBP)-tagged full-length (FL) protein A (MBP-protA) was expressed in *Escherichia coli* and purified (Qiu et al., 2014) (Fig. 1A). Standard gel mobility shift experiments with a DIG-labeled $RNA1_{(50–118)}$ were performed to determine the RNA binding ability of MBP-protA at 27 °C, and the samples were subject to 1.0% agarose gel electrophoresis. As shown in Fig. 2B, MBP-protA did bind to RNA efficiently (lane 5), whereas the negative controls (MBP alone, bovine serum albumin [BSA], and boiled MBP-protA) exhibited no RNA binding ability (lanes 2–4).

Moreover, RNA probe competition assays were performed to test whether MBP-protA specifically bound to $RNA1_{(50–118)}$ (Fig. 2C). Briefly, the same amounts of DIG-labeled RNA probe and MBP-protA were used in the presence of increasing amounts of unlabeled competitors, such as $RNA1_{(50–118)}$ wt, Del or Mut, and yeast tRNA (Fig. 2C). Only $RNA1_{(50–118)}$ wt efficiently competed with DIG-labeled $RNA1_{(50–118)}$, showing that protein A binds to RNA1 specifically. We also revealed that protein A can directly bind to RNA2 by the gel mobility shift experiment in the presence of MBP-protA and DIG-labeled $RNA2_{(123–164)}$ (Fig. 2D, lane 2). Moreover, increasing the amount of unlabeled $RNA2_{(123–164)}$ competitor resulted in the gradual reduction of the bound RNA probe, indicating that RNA2 is specifically bound to protein A as does RNA1 (Fig. 2D, lanes 3–5).

We used $RNA1_{(50–118)}$ as the RNA probe for the subsequent assays. To further characterize the behavior of protein A when it binds to RNA1, we incubated progressively increasing amounts of MBP-protA (0.1–6 μM) with 20 nM of DIG-labeled $RNA1_{(50–118)}$