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Helicase ATPase activity of the Tobacco mosaic virus 126-kDa protein modulates replicase complex assembly

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ABSTRACT

Mutations disrupting helicase domain motifs of the *Tobacco mosaic virus* 126/183-kDa proteins were investigated for their effect on replicase function and assembly. These mutations inhibited virus replication but did not affect 126-kDa induced *N* gene resistance or RNAi suppression. However, *in vivo* expressed 126-kDa motif mutants yielded two distinct cytoplasmic phenotypes that correlated with ATPase activity. Specifically, ATPase active 126-kDa proteins produced small cytoplasmic bodies that resembled the ovoid granular-like bodies found early in virus infection while 126-kDa proteins defective in ATPase activity produced large tubule containing cytoplasmic bodies similar to those observed late in infection. Additional studies indicate that the helicase ATPase activity resides predominantly within monomer and dimer helicase forms and that motifs affecting ATPase activity induce alterations in helicase assembly. Combined these findings indicate that helicase ATPase activity modulates the progression of replicase complex assembly and maturation.

Introduction

Positive-strand RNA viruses, including those infecting plants and animals, all form host membrane-associated virus replication complexes (VRCs) (Egger et al., 2000; Lyle et al., 2002; Ortin and Parra, 2006; Schaad et al., 1997; Schwartz et al., 2002). These complexes are composed of both viral and host components and serve as sub-cellular modules, supporting viral genome expression and replication. In addition, viral encoded components of the VRC, specifically the replication proteins, can contribute to other viral processes including the suppression of host defenses, cellular reprogramming, and inter-cellular virus movement (Ding et al., 2004; Kawakami et al., 2004; Kubota et al., 2003; Ortin and Parra, 2006; Whitham et al., 1996). This diversity of functions suggests the presence of specific mechanisms that modulate replicase function and VRC formation.

Similar to other positive-strand RNA viruses, *Tobacco mosaic virus* (TMV) replicates in close association with ER-derived host membranes (Heinlein et al., 1998; Más and Beachy, 1999). The genome of TMV encodes two co-terminal 5′ proximal open reading frames: a 126-kDa protein terminated by an amber stop codon and a readthrough 183-kDa protein, occurring ~5% of the time, that encodes the RNA-dependent RNA polymerase domain (Goelet et al., 1982). Both 126- and 183-kDa proteins possess an N-terminal methyltransferase (MT) domain and a 126-kDa C-terminal helicase (HEL) domain (Fig. 1A). Biochemical characterizations have identified guanylyltransferase-like activity involved in viral RNA capping from the N terminus of the 126-kDa protein and NTPase and RNA-unwinding activity derived from the C-terminal helicase domain (Dunigan and Zaitlin, 1990; Erickson et al., 1999; Goregaoker and Culver, 2003; Merits et al., 1999). Both TMV replication proteins are also major components of the membrane-associated VRCs (Más and Beachy, 1999; Osman and Buck, 1996). Within infected cells, the 126- and 183-kDa proteins appear to form cytoplasmic amorphous and rope-like structures embedded within a ribosome-rich and membrane associated matrix containing the viral movement protein, host proteins and the host ER membrane system (dos Reis Figueira et al., 2002; Esau and Cronshaw, 1967; Hills et al., 1987; Más and Beachy, 1999; Saito et al., 1987). TMV derived VRCs also associate with cellular microtubules and microfilaments, potentially facilitating the intracellular trafficking of viral RNA (Heinlein et al., 1998; Liu et al., 2005; Más and Beachy, 1999). Although the molecular structure of the TMV VRC has not been determined, an immunopurified heterodimer consisting of 126- and 183-kDa proteins has been shown to exhibit both template-dependent and template-specific RNA polymerase activities (Watanabe et al., 1999), suggesting that functional replication resides within small order assemblies of these proteins. The various forms and functions of the TMV VRC indicate that it is a dynamic entity with unique functionalities potentially imparted by various structural configurations of the 126- and 183-kDa proteins.

Studies indicate that there are a diversity of mechanisms involved in the recruitment of cellular membranes and the assembly of functional

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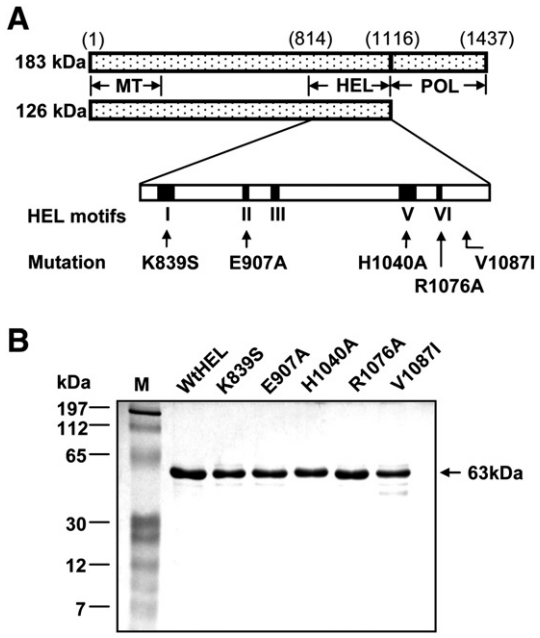

Fig. 1. Expression and purification of TMV helicase peptides. (A) Schematic representation of TMV 126-kDa and 183-kDa proteins indicating the locations of the helicase motifs and mutations. Amino acid numbers shown in parenthesis. MT, methyltransferase; HEL, helicase; POL, polymerase. (B) Nickel column purified helicase products (~15 μg each) resolved by SDS-PAGE gel and stained with Coomassie blue.

VRCs (Ahlquist et al., 2003; Buck, 1999; Nagy and Pogany, 2008; Strauss and Strauss, 1994; van der Heijden and Bol, 2002). For example, some viral replication proteins, such as *Flock house virus* protein A or *Brome mosaic virus* (BMV) protein 1a encode membrane spanning or amphipathic α-helices respectively that directly promote associations with cellular membranes (Liu et al., 2009; Miller and Ahlquist, 2002). Alternatively, replicase–membrane interactions can occur indirectly through interactions with host encoded transmembrane proteins as appears to be the case for TMV whose replication proteins associate with two integral membrane proteins, TOM1 and TOM3, that are required for efficient virus replication (Yamanaka et al., 2000). How these components coordinate with the host membrane system in the assembly of an active VRC is still unclear. For TMV, expression of the 126-kDa protein in the absence of other viral components is sufficient to direct the assembly of ER-associated complexes, suggesting that the 126-kDa protein plays a key role in recruiting and organizing viral and host components within the VRC (dos Reis Figueira et al., 2002; Liu et al., 2005).

Using the yeast two-hybrid system, a self-interaction within the helicase domain of the TMV 126-kDa protein has been identified (Goregaoker and Culver, 2003; Goregaoker et al., 2001). Disruption of this interaction by mutagenesis affects virus replication (Goregaoker et al., 2001). Helicase domains have been widely reported to be encoded by many RNA viruses and are generally considered to function in unwinding RNA duplexes formed during virus replication and the removal of secondary structures from RNA templates via NTP hydrolysis (Kadri and Haenni, 1997). Helicases can be classified into distinct superfamilies defined by amino acid sequence similarity (Gorbalenya et al., 1989; Gorbalenya et al., 1990; Singleton et al., 2007). The TMV RNA helicase belongs to superfamily one (SF1) and contains five conserved motifs (I, II, III, V, and VI) (Fig. 1A). Interestingly, the TMV 126-kDa protein and its helicase domain are implicated in the regulation of several host responses including cell to cell movement, the elicitation of *N* gene resistance, the suppression of host mediated RNAi defenses and the development of symptoms (Abbink et al., 2001; Bilgin et al., 2003; Ding et al., 2004; Erickson et al., 1999; Goregaoker et al., 2001; Hirashima and Watanabe, 2001; Kubota et al., 2003; Padgett and Beachy, 1993; Padmanabhan et al., 2005). The multi-functional nature of this domain suggests that its contributions in VRC assembly and function are likely to be highly regulated.

In this study, we investigated the functional effect of the TMV 126-kDa domain on replicase function and VRC assembly. Point mutations within the well-conserved helicase motifs (I, II, V, VI) were found to disrupt either ATPase and/or RNA binding activities as well as inhibit virus replication. However, these mutations did not disrupt the ability of the replication proteins to induce *N* gene resistance or RNAi suppression. Furthermore, biochemical and cellular analyses demonstrated that mutant helicases also maintained their ability to self-associate, indicating that the overall structural features of the replication proteins are not significantly altered by the mutations. However, disruption of ATPase activity but not RNA binding correlated with the inability of the 126-kDa protein to form wild-type-like early infection VRCs, suggesting an essential role for helicase ATPase activity in regulating VRC maturation. Additional structural studies indicate ATPase activity resides within the monomer and dimer forms of the helicase, suggesting that lower order replication protein aggregates function in active virus replication and coordinate VRC maturation.

Results

Expression and purification of recombinant helicase peptides

Sequence comparisons with other SF1 helicases show the TMV helicase domain encodes five conserved motifs (I, II, III, V, and VI) involved in ATP hydrolysis and RNA binding. To characterize the effect of these activities on VRC assembly, mutations were used to disrupt individual helicase motifs. Specific mutations included: K839S in motif I, previously shown to inhibit ATPase activity (Lewandowski and Dawson, 2000); E907A within motif II; H1040A in motif V; and R1076A within motif VI (Fig. 1A). Individual mutation sites were selected based on their conservation within SF1 motifs (Caruthers and McKay, 2002). An additional mutation, V1087I, located outside of the helicase motifs and shown to have little effect on virus infection was used as a control (Goregaoker et al., 2001; Padmanabhan et al., 2005). Recombinant histidine-tagged polypeptides covering the entire TMV helicase domain (amino acids 519–1116, ~63 kDa) of the TMV 126-kDa protein were purified on Ni^{+2} affinity purification columns under non-denaturing conditions and visualized by SDS-PAGE (Fig. 1B).

Characterization of ATPase, RNA binding and replication activities of TMV helicase motif mutants

The effect of each mutation on helicase ATPase and RNA binding activities was determined. Wild-type and the control V1087I proteins were similarly capable of ATP hydrolysis and RNA binding (Fig. 2A and B). Helicase motifs I and II (Walker A and B) contain residues that interact with MgATP/MgADP and promote hydrolysis (Caruthers and McKay, 2002). Mutations made in these motifs would predictably disrupt MgATP/MgADP binding and block ATPase activity. Specifically, the conserved K residue within motif I interacts with the phosphates of MgATP/MgADP while the conserved E residue in motif II coordinates Mg^{+2}. Both motif I (K839S) and II (E907A) mutations disrupted helicase ATPase activity (Fig. 2A). These mutations also displayed significant reductions in the ability to bind single-stranded RNA (Fig. 2B), suggesting coordination between ATPase activity and RNA binding. Within motif VI a conserved R residue interacts with the nucleotide phosphate. Disruption of this motif VI residue by mutation R1076A inhibited ATP hydrolysis as well as RNA binding activity (Fig. 2A and B). Thus, the coordination of ATPase activity and RNA binding also occurs within motif VI. Residues within motif V form an extended network of interactions between the different motifs. However, the targeted motif V H1040 residue would predictably interact only with nucleic acid. Consistent with this