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Association of the *Tobacco mosaic virus* 126 kDa replication protein with a GDI protein affects host susceptibility

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ABSTRACT

An interaction between the *Tobacco mosaic virus* (TMV) 126 kDa replication protein and a host-encoded Rab GDP dissociation inhibitor (GDI2) was identified and investigated for its role in infection. GDI proteins are essential components of vesicle trafficking pathways. TMV infection alters the localization of GDI2 from the cytoplasm to ER-associated complexes. Partial silencing of GDI2 results in significant increases in the number of TMV infection foci observed in inoculated tissues. However, GDI2 silencing does not affect TMV accumulation at the infection site, cell-to-cell movement, or susceptibility of the host to mechanical inoculation. Furthermore, increases in the number of successful infection foci were specific to TMV and correlated with the appearance of vesicle-like rearrangements in the vacuolar membrane. Tissue infiltrations with brefeldin A, an inhibitor of vesicle trafficking, also enhanced host susceptibility to TMV. Combined these findings suggest that the 126 kDa–GDI2 interaction alters vesicle trafficking to enhance the establishment of an infection.

Introduction

For simple positive-strand RNA viruses one essential step in the establishment of an infection involves the association of replication-associated components with elements of the host's endomembrane system (Netherton et al., 2007; Miller and Krijnse-Locker, 2008). The successful completion of this step is generally recognizable by the formation of membrane associated virus replication complexes (VRCs). While the form and cellular location of these membrane complexes can vary dramatically between different viruses, they all function to concentrate replication factors and mask nascent viral RNA synthesis from cellular defenses. To date, a number of virus and host components have been linked to the membrane rearrangements associated with VRC assembly (Salonen et al., 2005; Netherton et al., 2007; Miller and Krijnse-Locker, 2008). For example, the 3A protein of Poliovirus, a member of the *Picornaviridae*, uses the GTPase cellular secretory element Arf1 and its guanine exchange factor GBF1 to selectively recruit phosphatidylinositol-4-kinase IIIβ, which catalyzes the production of phosphatidylinositol-4-phosphate (PI4P) enriched membrane lipids (Hsu et al., 2010). PI4P enriched membranes subsequently attract the poliovirus 3D polymerase protein, initiating VRC assembly and virus replication. In contrast, the 1a protein of *Brome mosaic virus* (BMV), type member of the *Bromoviridae*, contains domains that include an amphipathic α-helix that mediate both membrane and self interactions (Liu et al., 2009). The self-assembly of 1a is thought to produce a shell-like structure that combined with its ability to associate with membranes produces the spherule-shaped invaginations that are characteristic of the BMV VRC. From these studies it is evident that the structural make-up and physiology of VRC associated endomembrane systems are reprogrammed during infection. Knowledge as to how these endomembrane systems are altered by the virus is thus essential to understanding the infection process.

In this study, we utilized *Tobacco mosaic virus* (TMV) as a model positive-strand RNA virus to identify and investigate the role of a Rab GDP dissociation inhibitor (GDI) in the establishment of infection. TMV is the type member of the genus *Tobamovirus* and encodes two replication proteins (126 and 183 kDa) as well as a coat protein and movement protein (Goelet et al., 1982). Both replication proteins contain methyltransferase and helicase domains. The 183 kDa replication protein contains an additional RNA-dependent RNA-polymerase domain that is produced via the read-through of an amber stop codon at the end of the 126 kDa protein (Pelham, 1978). Early in infection both 126 and 183 kDa proteins associate with the endoplasmic reticulum (ER), forming small membrane associated cytoplasmic VRCs (Heinlein et al., 1998; Mas and Beachy, 1999; dos Reis Figueira et al., 2002). As the infection progresses these smaller VRCs condense to form larger ER-derived membrane structures known as viroplasms or X-bodies (Esau and Cronshaw, 1967; Hills et al., 1987; Saito et al., 1987; Mas and Beachy, 1999). Evidence

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suggests that the 126 kDa replication protein is primarily responsible for the formation of these bodies as its expression alone results in the formation of both early and late stage VRC-like cytoplasmic bodies (dos Reis Figueira et al., 2002; Wang et al., 2010). Association of the 126 kDa replication protein with the hosts endomembrane system is thought to occur via an interaction with the transmembrane protein, TOM-1 (tobamovirus multiplication 1) (Yamanaka et al., 2000). TOM-1 and the closely related TOM-3 protein are required for virus replication and have been identified as possible membrane tethers for replication complexes (Yamanaka et al., 2002). Interestingly, TOM proteins have been shown to predominantly localize to the plant cell vacuolar membrane, suggesting that either these proteins localize differently during infection or that the vacuolar membrane plays a role in infection (Hagiwara et al., 2003).

Using a yeast two-hybrid approach we identified an interaction between the helicase domain of the TMV 126 kDa protein and the Arabidopsis encoded Rab GDP dissociation inhibitor protein 2 (GDI2). GDI proteins regulate the activity of Rab proteins and are essential components of vesicle trafficking within the cell. Rab proteins are members of the Ras superfamily of small GTPases that function to regulate intracellular vesicle trafficking pathways (Pfeffer, 2003; Barr, 2009). GDI proteins bind to Rab proteins in their inactive, GDP-bound state and recycle them to their donor membrane for use in further vesicle trafficking (Seabra and Wasmeier, 2004; Ali and Seabra, 2005). There are 57 identified Rab proteins in *Arabidopsis thaliana*, but only two identified GDI proteins (Bock et al., 2001; Pereira-Leal and Seabra, 2001). Both Arabidopsis GDI proteins share 95% identity and show similarly high levels of identity with GDI proteins in tomato, 82%, and tobacco, 84%. This level of homology suggests that these proteins are functionally conserved within a range of TMV hosts.

Since GDI proteins play an essential role in vesicle trafficking and because TMV replication occurs on vesicle-like membrane bodies, the interaction between GDI2 and the TMV 126 kDa replication protein was further investigated. Studies revealed that TMV infection alters the localization of GDI2 from the cytoplasm to ER-associated membrane bodies of similar size and shape to those produced during infection. Interestingly, silencing GDI2 prior to infection significantly increased the number of TMV infection foci produced upon inoculation, indicating that GDI2 regulates factors involved in host susceptibility. This enhanced susceptibility was specific to TMV and correlated with the appearance of vesicle-like rearrangements in the vacuolar membrane. Combined, these findings suggest that the TMV–GDI2 interaction modulates factors within the cell's vesicle transport system that contribute to the infection process.

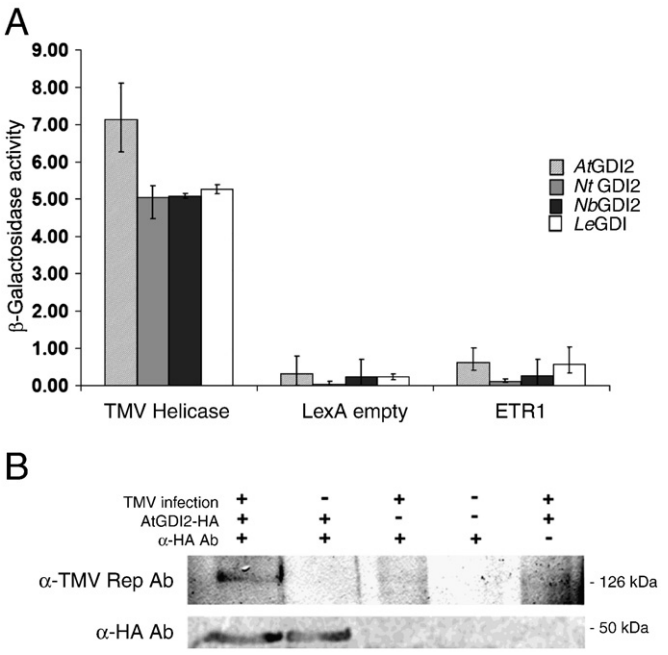

Fig. 1. Interaction between the TMV 126 kDa replication protein and GDI2. (A) Quantitative β-galactosidase assays displaying an interaction between the 126 kDa helicase domain (a.a. 814–1116) and the GDI proteins from Arabidopsis (*At*GDI2), *N. tabacum* (*Nt*GDI2), *N. benthamiana* (*Nb*GDI2) and tomato (*Le*GDI2). The ethylene receptor component ETR1 is used as a non-interacting control. (B) Co-immunoprecipitation of *At*GDI2-HA and the full-length TMV 126 kDa replication protein within TMV-infected tissues.

Results

Rab GDP dissociation inhibitor 2 (GDI2) interacts with the TMV 126 kDa protein

The helicase domain of the TMV 126/183 kDa replication proteins, amino acids 814–1116, was used in a two-hybrid assay to probe an *Arabidopsis thaliana* ecotype Nossen cDNA library for replicase interacting proteins. Of several interacting *Arabidopsis* clones, three were found to encode the C-terminal 110 amino acids of *At*GDI2 (At3g59920). To further confirm this interaction the full-length 445 amino acid (49.5 kDa) *At*GDI2 open reading frame (ORF) was cloned from the highly TMV susceptible Arabidopsis ecotype Shahdara (Dardick et al., 2000) and demonstrated via two-hybrid assay to interact with the viral helicase domain (Fig. 1A). Subsequent sequence comparisons revealed *At*GDI2 shares significant sequence identity with GDIs found in other important TMV hosts including *Solanum lycopersicum* (82%; TIGR gene index TC162880), *Nicotiana tabacum* (84%), and *Nicotiana benthamiana* (84%). Full-length GDIs cloned from these hosts were also found to interact with the TMV helicase domain via the two-hybrid assay (Fig. 1A), suggesting that this interaction is conserved among diverse TMV hosts.

To confirm the GDI2–TMV helicase interaction occurs within the context of an infection the *At*GDI2 ORF was fused to the hemagglutinin (HA) epitope and cloned into an *Agrobacterium* expression vector to create p35S::HA*At*GDI2. Agroinfiltration of p35S::HA*At*GDI2 into TMV or mock infected leaf tissue was subjected to pull-down assays using anti-HA antibodies. Results indicated that complexes containing HA*At*GDI2 and the full-length TMV 126 kDa replication protein were recovered only from tissues that were both p35S::HA*At*GDI2 infiltrated and TMV infected (Fig. 1B). Thus, HA*At*GDI2 is capable of interacting with the full-length 126 kDa replicase protein within the context of an active virus infection.

TMV infection alters AtGDI2-GFP localization

The effects of a TMV infection on the localization and accumulation of GDI2 were investigated using 35S::*At*GDI2-GFP transgenic *N. benthamiana* plants expressing the *At*GDI2 ORF fused to GFP. Low magnification fluorescent microscopy indicated that within uninfected tissues *At*GDI2-GFP fluorescence is localized to the cytoplasm of epidermal leaf cells (Fig. 2A). This is consistent with the localization of related GDI proteins and their role in vesicle transport (Ullrich et al., 1993; Seabra and Wasmeier, 2004). In contrast, we noted that within TMV-infected tissues *At*GDI2-GFP derived fluorescence was substantially altered, suggesting that infection changes either the localization or accumulation of the *At*GDI2-GFP protein (Fig. 2A). Western immunoblots for the detection of GFP were used to monitor the accumulation of *At*GDI2-GFP in both mock and TMV-infected tissues. Analysis of two independent transgenic 35S::*At*GDI2-GFP lines indicated no noticeable difference in the accumulation of *At*GDI2-GFP in either mock or infected tissues (Fig. 2B). Higher magnification confocal microscopy studies with the addition of Rhodamine B hexyl ester, an ER/mitochondria stain (Rashid and Horobin, 1990; Terasaki