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Identification of domains in p27 auxiliary replicase protein essential for its association with the endoplasmic reticulum membranes in *Red clover necrotic mosaic virus*

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ABSTRACT

Positive-strand RNA viruses require host intracellular membranes for replicating their genomic RNAs. In this study, we determined the domains and critical amino acids in p27 of *Red clover necrotic mosaic virus* (RCNMV) required for its association with and targeting of ER membranes in *Nicotiana benthamiana* plants using a C-terminally GFP-fused and biologically functional p27. Confocal microscopy and membrane-flotation assays using an *Agrobacterium*-mediated expression system showed that a stretch of 20 amino acids in the N-terminal region of p27 is essential for the association of p27 with membranes. We identified the amino acids in this domain required for the association of p27 with membranes using alanine-scanning mutagenesis. We also found that this domain contains amino acids not critical for the membrane association but required for the formation of viral RNA replication complexes and negative-strand RNA synthesis. Our results extend our understanding of the multifunctional role of p27 in RCNMV replication.

Introduction

The replication of positive-strand RNA viruses is performed by viral replication complexes that consist of viral RNA-dependent RNA polymerase (RdRp), viral auxiliary proteins, host-encoded proteins, and viral RNAs on intracellular membranes in infected cells (Ahlquist et al., 2003; den Boon et al., 2010; Nagy and Pogany, 2012). Intracellular membranes utilized by plant RNA viruses differ, and include those of the endoplasmic reticulum (ER), chloroplasts, vacuoles, peroxisomes, and mitochondria (Ahlquist et al., 2003; Nagy and Pogany, 2008; Ritzenthaler and Elamawi, 2006; Salonen et al., 2005). Viral proteins play essential roles in targeting viral replication complexes to the membranes and induce morphological alterations of the membranes (den Boon et al., 2010; Miller and Krijnse-Locker, 2008). For example, the 1a protein of *Brome mosaic virus* (BMV) localizes to ER membranes, induces vesicular ER invaginations or spherules, and recruits RNA templates to the membranes together with 2a RdRp (den Boon et al., 2010; Liu et al., 2009; Schwartz et al., 2002, 2004; Wang et al., 2005). The auxiliary replicase protein p33 of the tombusviruses (*Tomato bushy stunt virus* and *Cucumber necrosis virus*) recruits a replicon RNA and p92 RdRp into the peroxisomal membranes in yeast, a model host for the study of virus replication (McCartney et al., 2005; Panavas et al., 2005). The replicase protein p36 of *Carnation Italian ringspot virus* (CIRV), another member of the tombusviruses, localizes to the outer mitochondrial membrane and induces the formation of multivesicular bodies (Hwang et al., 2008; Rubino et al., 2001; Weber-Lotfi et al., 2002). Turnip yellow mosaic virus 140 K replication protein mediates the targeting of another 66 K replication protein with RdRp motif to the chloroplast envelope (Prod'homme et al., 2003). Domains essential for membrane association have been identified in the replication proteins of several viruses, including BMV 1a (den Boon et al., 2001; Liu et al., 2009; Schwartz et al., 2002), p33 and p36 of the tombusviruses (Hwang et al., 2008; Navarro et al., 2004; Panavas et al., 2005), tobacco etch virus 6 K protein (Schaad et al., 1997), tobacco mosaic virus 126 kDa protein (dos Reis Figueira et al., 2002), tomato ringspot nepovirus NTB-VPg and X2 proteins (Zhang et al., 2005; Zhang and Sanfaçon, 2006), poliovirus 2C and 3AB proteins (Echeverri and Dasgupta, 1995; Teterina et al., 1997; Towner and Semler, 1996), Hepatitis C virus (HCV) nonstructural proteins NS5A and NS5B (Brass et al., 2002; Schmidt-Mende et al., 2001), and flock house virus protein A (Van Wynsberghe et al., 2007).

Red clover necrotic mosaic virus (RCNMV) is a positive-sense single-stranded RNA plant virus with a bipartite genome and

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a member of the genus *Dianthovirus* in the family *Tombusviridae*. Both genomic RNAs lack a 5′ cap structure (Mizumoto et al., 2003) and a 3′ poly(A) tail (Lommel et al., 1988; Mizumoto et al., 2003; Xiong and Lommel, 1989). RNA1 (3.9 kb) encodes N-terminally overlapping replicase proteins, a 27 kDa protein (p27) and an 88 kDa protein (p88). p88 contains an RdRp motif (Koonin, 1991) produced by programmed-1 ribosomal frameshifting (Kim and Lommel, 1994; Tajima et al., 2011; Xiong et al., 1993) and is required in *cis* for RNA1 replication (Okamoto et al., 2008). RNA1 also encodes a 37 kDa coat protein that is translated from subgenomic RNA (Zavriev et al., 1996). RNA2 (1.5 kb) encodes a 35 kDa movement protein (MP) that is required for cell-to-cell movement in plants (Xiong et al., 1993).

The auxiliary replicase protein p27 of RCNMV, together with p88 and some host proteins, forms the 480 kDa viral replicase complex associated with ER membranes in RCNMV-infected cells (Mine et al., 2010a). Assembly of the 480 kDa complexes requires both p27–p27 and p27–p88 interactions, in which the C-terminal region of p27 and the nonoverlapping region unique to p88 are involved (Mine et al., 2010b). p27 also plays an essential role in the recruitment of RNA2 to ER membranes via binding to a Y-shaped RNA element (YRE) in the 3′ noncoding region of RNA2 (Iwakawa et al., 2011). The domains and critical amino acids required for RNA binding were mapped to the central and the C-terminal regions of p27 (Hyodo et al., 2011). A preceding study with confocal microscopy using N-terminally green fluorescent protein (GFP)-fused p27 (GFP-p27) and p88 (GFP-p88) revealed that both proteins colocalize to the cortical and cytoplasmic ER, and that p27 causes ER membrane restructuring and proliferation (Turner et al., 2004). However, the relationship between the protein localization and the formation of a functional replication complex remains elusive.

In this study, we determined the domains and critical amino acids in p27 required for its association with and targeting of ER membranes using a C-terminally GFP-fused p27 (p27-GFP) that supports viral RNA replication in the presence of p88. Confocal microscopy and membrane-flotation assays revealed that the membrane association of p27 is mediated by a stretch of 20 amino acids located in the N-terminal region of p27 (amino acids 31–50) and that the 20 amino acid sequence is sufficient for targeting nonviral GFP to ER membranes. Mutations that impeded the membrane association of p27 compromised the formation of RCNMV RNA replication complexes and negative-strand RNA synthesis.

Results and discussion

p27-GFP supports RCNMV RNA replication in the presence of p88 and localizes to ER membranes

First, to obtain a biologically functional GFP-fused p27, we constructed N-terminally GFP-fused p27 (GFP-p27) and C-terminally GFP-fused p27 (p27-GFP) (Fig. 1A), and tested their ability to support the replication of RNA2 by coexpression with p88 and RNA2 using an *Agrobacterium*-mediated expression system (agroinfiltration) in *Nicotiana benthamiana* (Takeda et al., 2005). Accumulations of viral proteins and RNAs were analyzed by western and northern blotting methods, respectively, at 2 days after infiltration (dai). p27-GFP, but not GFP-p27, accumulated and supported the accumulation of both negative and positive-strand RNA2 (Fig. 1B). These results indicate that p27-GFP is biologically functional. In contrast, GFP-p27 seems to be unstable and non-functional in our experimental system. The mRNA levels of GFP-p27 and p27-GFP were similar when assessed by RT-PCR using total RNA isolated from *Agrobacterium*-infiltrated leaves expressing GFP-p27 or p27-GFP in the presence of p88 and RNA2 (data not shown). Moreover, C-terminally DsRed-monomer (DRm)-fused p27 (p27-DRm) was more stable than N-terminally DRm-fused p27 (DRm-p27) and only p27-DRm supported RCNMV RNA replication (K. Kusumanegara and T. Okuno, unpublished data). Therefore, we used p27-GFP in further experiments.

In a previous study, N-terminally GFP-fused p27 was successfully used to investigate intracellular localization of p27 (Turner et al., 2004). The success could be attributed to the experimental system, in which the fusion protein was expressed from RCNMV RNA1 or a viral vector, or expressed from a plasmid vector via microprojectile bombardment.

We investigated the subcellular localization of p27-GFP in the agroinfiltrated *N. benthamiana* leaves using a confocal laser scanning microscopy. The fluorescence of p27-GFP was observed as large aggregates in most of the cells observed, and these fluorescent areas merged well with those of an ER marker at 2 dai (Fig. 1C, upper panels). The fluorescence of p27-GFP was also observed to merge with that of the ER marker in the cortical and perinuclear regions but was not observed inside the nucleus (data not shown). The patterns of observed fluorescence of p27-GFP were similar at 2, 3, and 4 dai (data not shown). In contrast, the fluorescence of free GFP was observed in the cytoplasm and these fluorescent areas did not coincide with those of an ER marker (Fig. 1C, lower panels). The fluorescence of free GFP was also observed inside the nucleus of leaf epidermal cells (Fig. 1C, lower panels, and data not shown). These observations suggest that p27-GFP colocalizes with the ER membranes and that p27-GFP disrupts a normal reticular structure of cortical ER by inducing membrane restructuring and proliferation, as reported previously by Turner et al. (2004).

Next, we investigated the subcellular localization of p27-GFP in the presence of p88 and RNA2. At 2 dai, small fluorescent punctates were observed along ER filaments in most cells and large aggregates were barely detected (Fig. 1C, middle panels). Later at 3 and 4 dai, larger aggregates associated with the proliferated ER adjacent to the nucleus were detected in many cells (data not shown). Transition of localization pattern of p27-GFP is similar to that of the fusion protein of RCNMV MP and GFP (MP-GFP) in *N. benthamiana* epidermal cells and protoplasts infected with the recombinant virus expressing the MP-GFP (Kaido et al., 2009). The small punctates might be a progressive form of RNA replication complexes. The significance of the difference between the small punctates and large aggregates was not further addressed in this study.

Domains in p27 essential for its subcellular localization and association with the ER membranes

To determine domain(*s*) of p27 essential for its subcellular localization and association with the ER membranes, we expressed the GFP-fused N-terminal half of p27 (p27N1–113-GFP) and GFP-fused C-terminal half of p27 (p27N114–236-GFP) together with an ER marker in *N. benthamiana* leaves and observed their subcellular localization, as described above. Fluorescence of p27N1–113-GFP was observed as small aggregates and punctate spots in the cytoplasm and merged well with the ER marker (Fig. 2C). Expression of p27N1–113-GFP similar to p27-GFP induced morphological alteration of cortical ER, although the size of aggregates appeared to be smaller than that of the aggregates induced by wild-type p27-GFP (Fig. 2B and C). In contrast, expression of p27N114–236-GFP, similar to wild-type free GFP, appeared not to induce the morphological alteration of ER. In addition, similar to wild-type free GFP, the fluorescence of p27N114–236-GFP dispersed in the cytoplasm and barely merged with the fluorescence of the ER marker (Fig. 2A and D). These results indicate that the N-terminal half of p27 is responsible for its membrane association.