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Characterization of dominant-negative and temperature-sensitive mutants of tombusvirus replication proteins affecting replicase assembly

Kunj B. Pathak, Zuodong Jiang, Verena Ochanine, Monika Sharma, Judit Pogany, Peter D. Nagy*

Department of Plant Pathology, University of Kentucky, Lexington, KY 40546, USA

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ABSTRACT

The assembly of the viral replicase complex (VRC) on subcellular membranes is a key step in the replication process of plus-stranded RNA viruses. In this work, we have identified lethal and temperature sensitive (ts) point mutations within the essential p33:p33/p92 interaction domain of p33 and p92 replication proteins of *Cucumber necrosis virus*, a tombusvirus. Mutations within the p33:p33/p92 interaction domain also affected viral RNA recombination in yeast model host. An *in vitro* approach based on yeast cell free extract demonstrated that several p33 and p92 mutants behaved as dominant-negative during VRC assembly, and they showed reduced binding to the viral (+)RNA and affected activation of the p92 RdRp protein, while they did not directly influence (−) or (+)-strand synthesis. Overall, the presented data provide direct evidence that the p33:p33/p92 interaction domains in p33 and p92 are needed for the early stage of virus replication and also influence viral recombination.

Introduction

Positive-stranded (+)RNA viruses infecting eukaryotic hosts share many fundamental processes during their replication. The similarities include (i) the participation of (+)RNA in translation to produce essential viral replication proteins as well as in genome replication as a template; (ii) the use of (−)RNA as template for production of (+)-strand RNA progeny in an asymmetric manner; (iii) the need for virus-specific replicase complexes (VRC), which are associated with intracellular membranes; and (iv) the use of subverted host proteins to facilitate viral replication (Bartenschlager et al., 2010; den Boon and Ahlquist, 2010; Miller and Krijnse-Locker, 2008; Nagy and Pogany, 2011; Novoa et al., 2005; Salonen et al., 2005; Shi and Lai, 2005). Indeed, all (+)RNA viruses likely depend on or require host factors to replicate their genomic RNAs in infected cells (Bartenschlager et al., 2010; den Boon et al., 2010; Li and Nagy, 2011; Nagy and Pogany, 2010, 2011, 2012; Nagy et al., 2011; Shi and Lai, 2005). Due to the above similarities, our understanding of replication of (+)RNA viruses has advanced significantly in the last decade based on a small number of model viruses, including tombusviruses, such as *Tomato bushy stunt virus* (TBSV) and *Cucumber necrosis virus* (CNV).

Similar to other (+)RNA viruses, the genomic RNA of TBSV and other tombusviruses is replicated by the membrane-bound VRC (Nagy, 2008; Nagy and Pogany, 2010, 2011; White and Nagy, 2004). The tombusvirus VRC contains the viral-coded p33 and p92 replication proteins and host-coded proteins, including glyceraldehyde-3-phosphate dehydrogenase, the heat shock protein 70 chaperones (Hsp70), pyruvate decarboxylase (Serva and Nagy, 2006), Cdc34p E2 ubiquitin conjugating enzyme, Ded1 DEAD-box RNA helicase, eukaryotic translation elongation factor 1A (eEF1A), eukaryotic translation elongation factor 1Bgamma (eEF1Bγ), Pex19p shuttle protein and the Vps23p adapter ESCRT protein (Barajas et al., 2009; Barajas and Nagy, 2010; Huang and Nagy, 2011; Kovalev et al., 2012; Li et al., 2008, 2009, 2010; Nagy and Pogany, 2012; Pathak et al., 2008; Sasvari et al., 2011; Serva and Nagy, 2006; Wang and Nagy, 2008). The roles of several of these proteins in tombusvirus replication have been studied in some detail (Nagy and Pogany, 2010; Pogany et al., 2008; Wang and Nagy, 2008; Wang et al., 2009a, b). In addition to the above protein components, the tombusviral VRC also contains the viral plus-strand RNA, which likely serves as an assembly platform for the proteins (Panaviene et al., 2005; Pathak et al., 2012; Pogany et al., 2008). Due to the central role of the assembly of the VRC in virus replication, detailed studies have been conducted with several model RNA viruses to identify protein and RNA factors affecting VRC assembly and to characterize interactions

* Corresponding author at: Department of Plant Pathology, University of Kentucky, 201F Plant Science Building, Lexington, KY 40546, USA.
Fax: +859 323 1961.
E-mail address: pdnagy2@uky.edu (P.D. Nagy).

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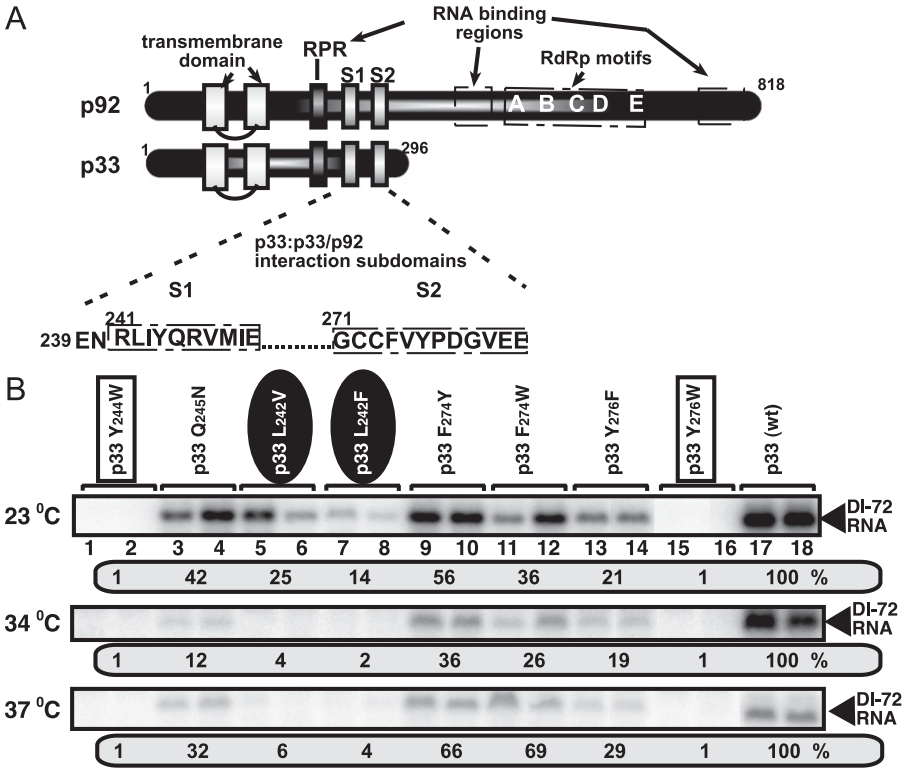

Fig. 1. Mutations in p33:p33/p92 interaction domain in p33 replication co-factor inhibits TBSV repRNA replication in yeast. (A) Cartoons of p33 (1–296 aa) and p92 (1–818 aa, a readthrough product of the p33 ORF, sharing the N-terminal 296aa with p33) replication proteins depicting various domains, such as S1 and S2 protein-protein interaction subdomains, an RNA binding (RPR) and a transmembrane domains. The sequences of S1 subdomain (241–251 aa) and S2 subdomain (271–283 aa) are shown. Quick-change mutagenesis approach was used to introduce specific mutations (see *Materials and methods*) into the S1/S2 subdomains. (B) Northern blot analysis of TBSV repRNA accumulation in BY4741 yeast cells co-expressing one of the mutated CNV His_6-tagged p33s, wt His_6-p92 and DI-72 repRNA. The names of the S1/S2 subdomain mutants indicate the mutations present in His_6-p33. Yeast was grown at three different temperatures to identify temperature-sensitive mutants (shown in black ovals). Nonfunctional p33 mutants are boxed, while the remaining mutants supported reduced repRNA accumulation. The control yeast expressed the wt His_6-tagged p33, and the replication level was taken as 100%. The 18S ribosomal RNA was used as a loading control (not shown). Quantification was done with Imagequant software. The experiments were repeated 2 times.

among the above factors (den Boon et al., 2010; Li and Nagy, 2011; Nagy and Pogany, 2012).

The assembly of the tombusviral VRC likely starts with specific interaction between the tombusviral (+)RNA and the p33 and $p92^{pol}$ proteins, which results in selection of the viral RNA for replication and the recruitment of the viral RNA to the site of replication [*i.e.*, the cytoplasmic surface of the peroxisomal membrane (McCartney et al., 2005; Panavas et al., 2005; Pathak et al., 2008)]. The specific binding of p33 to the TBSV (+)RNA requires the p33 recognition element (p33RE), which includes a C●C mismatch within a large, conserved stem-loop structure, denoted RII(+)-SL (Monkewich et al., 2005; Pogany et al., 2005). p33 and the overlapping $p92^{pol}$ contains an RNA-binding region (RPR domain, Fig. 1A) needed for binding to p33RE of TBSV (+)RNA (Pogany et al., 2005; Rajendran and Nagy, 2003). P33 and $p92^{pol}$ also contain two small p33:p33/p92 interaction domains, denoted S1 and S2 (Fig. 1A), which are also required for the specific recognition of p33RE *in vitro* (Rajendran and Nagy, 2004, 2006). The requirement for p33:p33/p92 interaction domain for specific viral RNA binding indicates that p33 and $p92^{pol}$ likely binds RNA as a dimer (or multimer). Because the N-terminal region of $p92^{pol}$ has the same sequence as p33, it has been proposed that $p92^{pol}$ might also be part of the above protein:RNA complex (Rajendran and Nagy, 2004, 2006). In addition to binding to both $p92^{pol}$ and to the TBSV (+)RNA, p33 also binds to host factors, such as Hsp70 (Nagy and Pogany, 2010), and it has peroxisomal targeting sequences and membrane-spanning domains that facilitate the association of p33 with peroxisomal membrane surfaces, the sites of TBSV replication (McCartney et al., 2005; Panavas et al., 2005). In spite of the above advances, our current understanding of the assembly of TBSV VRC and the factors involved are incomplete.

To further characterize the multifunctional nature of the p33 replication protein, in the first part of the paper, we identify mutants within the S1 and S2 subdomains of the p33:p33/p92 interaction domain that render the replication protein nonfunctional, temperature sensitive (ts) or dominant-negative. Testing the accumulation of TBSV RNA in yeast, a model host led to the identification of critical amino acid residues within the p33:p33/p92 interaction domain as well as generation of ts mutants, which supported TBSV replication only at the permissive temperature. Using temperature shift experiments, we demonstrated that p33:p33/p92 interaction is required at an early step in tombusvirus replication. Then, in the second part of the paper, we dissect the mechanism of the inhibitory effect of p33/p92 mutations on tombusvirus replication. We find that mutations within the p33:p33/p92 interaction domain reduced the efficiency of the *in vitro* assembly of the functional tombusvirus VRC, affected the *in vitro* activation of the p92 RdRp protein, and reduced the ability of p33 to bind to the viral (+)RNA. Altogether, the presented data provide direct functional roles for the p33:p33/p92 interaction domains in p33 and p92 that are required for replication of tombusviruses.

Results

Identification of critical amino acid residues within p33:p33/p92 interaction domain of p33 replication protein affecting tombusvirus replication in yeast

Previous works have defined the S1 and S2 subdomains of the p33:p33/p92 interaction sequence (Fig. 1A), which are known to be essential for TBSV replication and the assembly of the tombusvirus