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Kinetics and functional studies on interaction between the replicase proteins of *Tomato Bushy Stunt Virus*: Requirement of p33:p92 interaction for replicase assembly

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Abstract

The assembly of the functional replicase complex via protein:protein and RNA:protein interactions among the viral-coded proteins, host factors and the viral RNA on cellular membranes is a key step in the replication process of plus-stranded RNA viruses. In this work, we have characterized essential interactions between p33:p33 and p33:p92 replication proteins of *Tomato bushy stunt virus* (TBSV), a tombusvirus with a non-segmented, plus-stranded RNA genome. Surface plasmon resonance (SPR) measurements with purified recombinant p33 and p92 demonstrate that p33 interacts with p92 in vitro and that the interaction requires the S1 subdomain, whereas the S2 subdomain plays lesser function. Kinetic SPR analyses showed that binding of S1 subdomain to the C-terminal half of p33 takes place with moderate binding affinity in the nanomolar range whereas S2 subdomain binds to p33 with micromolar affinity. Using mutated p33 and p92 proteins, we identified critical amino acid residues within the p33:p92 interaction domain that play essential role in replication and the assembly of the tombusviral replicase. In addition, we show that interaction takes place between replication proteins of TBSV and the closely related *Cucumber necrosis virus* but not between TBSV and the more distantly related *Turnip crinkle virus*, suggesting that selective protein interactions might prevent the assembly of chimeric replicases carrying replication proteins from different viruses during mixed infections.

Keywords: Virus replication; RdRp; Protein–protein interactions; Surface plasmon resonance; Coimmunoprecipitation assay; Yeast host

Introduction

Plus-stranded RNA viruses of eukaryotes use viral replicases assembled on intracellular membranes to synthesize new viral RNA progenies. These replicase complexes contain viral RNA template(s) and viral- and host-coded proteins (Ahlquist et al., 2003; Buck, 1996; Noueiry and Ahlquist, 2003). Several studies have been conducted to dissect protein–protein and protein–RNA interactions that hold the protein and RNA factors together within the replicase complex. In case of *Brome mosaic virus* (BMV), the interaction between the viral-coded 1a and 2a replicase proteins has been demonstrated using coimmunoprecipitation and yeast two-hybrid assays (Kao et al., 1992; O'Reilly et al., 1995, 1997). Protein 1a also interacts with other 1a proteins, which might be important to bring two or more 1a proteins into complex with 2a proteins (O'Reilly et al., 1998; Schwartz et al., 2002). Overall, the 1a and 2a interactions are essential for BMV replication as confirmed in replication studies in plant protoplasts using selected BMV mutants (O'Reilly et al., 1998). Another example is the 126K protein of *Tobacco mosaic virus* (TMV), which interacts with other 126K proteins and with the 186K RNA-dependent RNA polymerase (RdRp) protein (Goregaoker et al., 2001; Watanabe et al., 1999). The interaction between 126K and 186K is essential for TMV replication, based on studies in protoplasts using temperature-sensitive 126K mutants (Goregaoker and Culver, 2003). Interaction between various replicase proteins has also been demonstrated for other plus-stranded RNA viruses, including poliovirus (Agol et al., 1999; Hope et al., 1997; Lyle et al., 2002; Racaniello and Ren, 1996), hepatitis C virus (reviewed by (Tellinghuisen and Rice, 2002), cucumo-

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viruses (Suzuki et al., 2003) and potyviruses (Schaad et al., 1997).

Tombusviruses are model plus-stranded RNA viruses of plants, which include *Tomato bushy stunt virus* (TBSV) and *Cucumber necrosis virus* (CNV). The single component tombusvirus genomic (g)RNA codes for five proteins of which only two, namely p33 and p92 are essential for viral replication (Russo et al., 1994; White and Nagy, 2004). Protein p92 is produced via ribosomal readthrough of the p33 stop codon and it includes the signature motifs of RdRp in its unique C-terminal domain (O'Reilly and Kao, 1998). The concentration of p92 is about 20-fold lower than p33 in infected plant cells (Scholthof et al., 1995), and ~10-fold less in yeast cells that efficiently replicate a model TBSV replicon (Panaviene et al., 2004). Importantly, both p33 and p92 proteins are present in highly active replicase preparations purified from yeast co-expressing CNV p33 and p92 proteins and DI-72 RNA (Panaviene et al., 2004, 2005), suggesting that they might be involved in viral RNA synthesis.

The purified tombusvirus replicase has been shown to synthesize complementary RNA on added plus- or minus-stranded TBSV RNA templates (Nagy and Pogany, 2000; Panaviene et al., 2004), demonstrating that it could accurately recognize terminal promoter sequences (Panavas et al., 2002a, 2002b), replication enhancers (Panavas and Nagy, 2003a, 2005; Panavas et al., 2003) and a replication silencer element (Pogany et al., 2003) during RNA synthesis. The significance of these cis-acting RNA elements for tombusvirus replication has been confirmed using plant protoplasts (*Nicotiana benthamiana* and cucumber) (Fabian et al., 2003; Panavas and Nagy, 2005; Panavas et al., 2003; Pogany et al., 2003; Ray and White, 1999, 2003) and yeast, a model host (Panavas and Nagy, 2003b). However, much less is known about the protein components of the replicase complex and the interactions between these proteins.

The p33 replication protein is likely part of the active replicase complex, because it is an essential co-factor in viral replication (Oster et al., 1998; Panaviene et al., 2003, 2004), co-purified with the active replicase fractions (Panaviene et al., 2004, 2005), and co-localized with p92 and the (−)-stranded viral RNA replication intermediates in cells (Panavas et al., 2005a). Previous studies have established that p33 has four different domains: (i) the N-terminal hydrophylic region that is essential for replication, albeit its function is yet unknown (Panavas et al., 2005a); (ii) the N-proximal hydrophobic membrane anchoring domain with two predicted trans-membrane helices, which is essential for targeting of p33 to the peroxisomal membrane surfaces, the sites of viral RNA replication (Navarro et al., 2004; Panavas et al., 2005a); (iii) the RNA binding region consisting of an arginine-proline rich motif (RPR motif, Fig. 1A), (Panaviene and Nagy, 2003; Panaviene et al., 2003; Rajendran and Nagy, 2003); (iv) the C-terminal p33:p33/p92 protein interaction domain that contains two nonoverlapping sites, termed S1 and S2 that can independently facilitate binding of p33 to p33 and p92 (Rajendran and Nagy, 2004). The significance of p33:p33 and p33:p92 interactions was confirmed in a model tombusvirus replication system in yeast by expressing p33 and p92 proteins carrying site-specific mutations within the region needed for protein interaction. The mutational studies in S1 and S2 revealed that tyrosine and arginine at positions 244 and 246 in p33 and p92 sequence are indispensable for viral RNA replication. However, it is not known whether their presence is required for p33:p33/p92 interaction and assembly of active replicase in vivo. This gap in knowledge is addressed in this present study.

To understand the proposed role of p33:p33 and p33:p92 interactions in the assembly of the tombusvirus replicase complex, in this paper, we studied the kinetics of protein interaction and the functional relevance of this interaction in the replicase assembly process. Surface plasmon resonance (SPR) analyses with purified recombinant proteins revealed that the S1 subdomain in p33 is a major contributor to p33:p33 and p33:p92 interactions in vitro. The S1 subdomain is also essential for in vivo p33:p92 interactions as demonstrated by S1 mutants in coimmunoprecipitation assay. Moreover, there is a good correlation between p33:p92 interaction, replicase assembly and replication levels, suggesting that viral protein interactions are important during tombusvirus replication. Also, SPR analysis showed good interaction between TBSV p33 and its closely related CNV p33 and p92 replication proteins, whereas the more distantly related p28 and p88 of *Turnip crinkle virus* (TCV) did not interact with TBSV p33 in vitro. These data promote the idea that the assembly of chimeric replicases carrying TBSV and TCV replication proteins are unlikely to take place in mixed infections.

Results and discussion

In vivo interaction between full length p33 and p92 replication proteins

Previous works have demonstrated interactions between p33 and p92 molecules in vitro and between N-terminally truncated p33 and p92 in yeast two-hybrid assay (Rajendran and Nagy, 2004). To examine interaction between full-length p33 and p92 in vivo, we expressed single (His_6) or double tagged (His_6 and FLAG) full-length p33 and p92 proteins in yeast as shown in Fig. 1B, in the presence of the replicon RNA to obtain functional replicase complexes (Panaviene et al., 2004).

Immunoprecipitation with anti-FLAG antibodies of His_6/FLAG-p92 resulted in co-purification of His_6-p33 (Fig. 1B, lane 3). The immunoprecipitated complex also showed replicase activity on added RNA templates in vitro (Fig. 1C, lane 3). Interestingly, the immunoprecipitated replicase complex contained ~3-fold more p33 than p92, suggesting that p33 is more abundant than p92 in the replicase complex. Similarly, immunoprecipitation with anti-FLAG antibodies of His_6/FLAG-p33 resulted in co-purification of His_6-p92 (Fig. 1B, lane 4) that showed replicase activity in vitro (Fig. 1C, lane 4). This replicase complex, which contained over 10-fold more p33 than p92, showed the highest replicase activity (Fig. 1C, lane 4). These data suggest that there is direct correlation