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A temperature sensitive mutant of heat shock protein 70 reveals an essential role during the early steps of tombusvirus replication

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ABSTRACT

By co-opting host proteins for their replication, plus-stranded RNA viruses can support robust replication and suppress host anti-viral responses. *Tomato bushy stunt virus* (TBSV) recruit the cellular heat shock protein 70 (Hsp70), an abundant cytosolic chaperone, into the replicase complex. By taking advantage of yeast model host, we demonstrate that the four-member *SSA* subfamily of *HSP70* genes is essential for TBSV replication. The constitutively expressed *SSA1* and *SSA2*, which are resident proteins in the viral replicase, can be complemented by the heat-inducible *SSA3* and/or *SSA4* for TBSV replication. Using a yeast strain carrying a temperature sensitive *ssa1*^{ts}, but lacking functional *SSA2/3/4*, we show that inactivation of Ssa1p^{ts} led to a defect in membrane localization of the viral replication proteins, resulting in cytosolic distribution of the viral proteins and lack of replicase activity. An in vitro replicase assembly assay with Ssa1p^{ts} revealed that functional Ssa1p is required during the replicase assembly process, but not during minus- or plus-strand synthesis. Temperature shift experiments from nonpermissive to permissive in *ssa1*^{ts} yeast revealed that the re-activated Ssa1p^{ts} could promote efficient TBSV replication in the absence of other *SSA* genes. We also demonstrate that the purified recombinant Ssa3p can facilitate the in vitro assembly of the TBSV replicase on yeast membranes, demonstrating that Ssa3p can fully complement the function of Ssa1p. Taken together, the cytosolic SSA subfamily of Hsp70 proteins play essential and multiple roles in TBSV replication.

Introduction

Due to limiting coding capacity of their genomes, plus-stranded (+)RNA viruses rely extensively on the host during their replication. These viruses hijack subcellular membranes and use the components of the host cells to make viral proteins and replicate the viral RNA. Moreover, they co-opt selected host proteins to facilitate viral genome replication (Ahlquist et al., 2003; Nagy, 2008; Noueiry and Ahlquist, 2003; Salonen et al., 2005; Shi and Lai, 2005). Indeed, recent genome-wide screens with *Brome mosaic virus* (BMV), *Tomato bushy stunt virus* (TBSV), Drosophila C virus, hepatitis C virus and West Nile virus revealed that more than 100 host proteins and many cellular pathways affected replication and infections by each (+)RNA virus (Cherry et al., 2005; Jiang et al., 2006; Krishnan et al., 2008; Kushner et al., 2003; Panavas et al., 2005b; Randall et al., 2007; Serviene et al., 2005). The functions of most of the identified host proteins during virus replication, however, are currently unknown.

TBSV, a small (+)RNA virus is used to dissect the roles of host proteins within the viral replicase, which is the key enzyme for viral genome replication. The tombusvirus replicase has been intensively characterized via proteomics approaches, in vitro replication assays and development of yeast as a model host (Li et al., 2008, 2009; Nagy, 2008; Nagy and Pogany, 2000; Panavas and Nagy, 2003; Panaviene et al., 2004; Pogany and Nagy, 2008; Serva and Nagy, 2006; Wang and Nagy, 2008). Previous work revealed that TBSV RNA replication depends on two viral-encoded proteins, namely the RNA-dependent RNA polymerase (p92^{pol} RdRp) and p33 replication co-factor, the key protein in recruitment of the viral RNA into replication (Monkewich et al., 2005; Pogany and Nagy, 2008; Pogany et al., 2008, 2005). The peroxisome membrane-bound TBSV replicase also contains 6-to-10 host proteins, which are likely involved in most activities of the replicase (Nagy and Pogany, 2006; White and Nagy, 2004). The identified host proteins within the tombusvirus replicase include heat shock protein 70 (Hsp70, coded by the constitutively expressed *SSA1* and *SSA2* genes in yeast) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, coded by *TDH2* and *TDH3* genes in yeast), which binds to the TBSV (-)RNA and affects plus-strand synthesis (Wang and Nagy, 2008). The replicase also contains translation elongation factor 1A (eEF1A), which binds to a *cis*-acting regulatory element in the TBSV (+)RNA as well as to p33 co-factor (Li et al., 2009). Another host-derived component is Cdc34p ubiquitin-conjugating enzyme, which ubiquitinates the p33 replication co-factor (Li et al., 2008). Down-regulation of these host factors inhibited, whereas their over-expression increased TBSV accumulation in yeast model host (Li et al., 2008; Serva and Nagy, 2006; Wang and Nagy, 2008) suggesting that

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they play significant roles in TBSV replication. In addition, Pex19p cytosolic transport protein binds transiently to the viral replication proteins as well as to the replicase complex, likely facilitating the transport of the replication proteins to the peroxisomal membranes, the site of replication (Pathak et al., 2008). The functions of the above host proteins within the viral replicase are currently under intensive investigations.

The host-coded Hsp70 chaperone family, which represents a major group among the heat shock proteins, and its co-chaperones have been suggested to promote replication of several (+)RNA and (-)RNA viruses (Brown et al., 2005; Dufresne et al., 2008; Nishikiori et al., 2006; Qanungo et al., 2004; Weeks and Miller, 2008). Based on the known cellular functions, Hsp70 and other chaperones were proposed to stimulate viral RdRp activity (Momose et al., 2002), and participate in the assembly of the viral replicase and enhance replication (Kampmueller and Miller, 2005; Tomita et al., 2003; Weeks and Miller, 2008). Plants infected by various plant viruses express cytosolic Hsp70 proteins at elevated levels, indicating that Hsp70 could play an important role during viral infections (Aparicio et al., 2005; Aranda et al., 1996; Whitham et al., 2003; Whitham et al., 2006). Moreover, a viral-encoded Hsp70-like protein is involved in the assembly of virions and cell-to-cell movement of closteroviruses (Alzhanova et al., 2001; Peremyslov et al., 1999). In addition, Hsp70-like proteins are involved in nuclear localization, genome replication and cell transformation of DNA viruses, cell entry, virion assembly and disassembly, envelope protein maturation, folding of capsid proteins, and viral transcription by various viruses (Mayer, 2005). Additional cellular chaperones, such as Hsp90 proteins or the J-domain containing Hsp40 proteins have also been shown to affect virus replication, including activation of reverse transcriptase for hepadnaviruses (Hu et al., 2004; Stahl et al., 2007; Tavis et al., 1998), or assembly of the BMV replicase (Tomita et al., 2003). Most of the above studies point toward Hsp70 and other cellular chaperones as major players during virus replication.

Host proteins could play different roles during tombusvirus replication. Currently, tombusvirus replication is divided into six sequential steps: RNA template selection by p33 replication protein; recruitment of the replication protein-viral RNA complex to the site of replication; assembly of the viral replicase complex; synthesis of viral progeny RNAs, including minus- and plus-strand synthesis; release of the newly made plus-strand viral RNAs from the replicase; and disassembly of the viral replicase (Nagy and Pogany, 2006). Hsp70 proteins might play multiple roles/functions in TBSV replication. Accordingly, the two cytosolic Ssa1p and Ssa2p Hsp70 proteins expressed constitutively in yeast have been shown to be recruited from the cytosol to the peroxisomal membrane (the site of TBSV replication) via interaction with the p33 replication co-factor (Serva and Nagy, 2006; Wang et al., 2009). Down-regulation or over-expression of Ssa1/2p in yeast resulted in reduced and elevated level of TBSV RNA accumulation, respectively, suggesting that these Hsp70 proteins are important for TBSV RNA replication (Serva and Nagy, 2006). These Hsp70 proteins are likely involved directly in replication since Ssa1/2p have been shown as components of the highly purified tombusvirus replicase complex (Serva and Nagy, 2006). Using a *HSP70* mutant yeast (*ssa1ssa2*), we found that the viral replication proteins remained cytosolic at an early time point, suggesting that Hsp70 is involved in subcellular localization of the viral replication proteins to intracellular membranes (Wang et al., 2009). A novel in vitro replication assay also showed that Ssa1/2p are essential for the assembly of the TBSV replicase (Pogany et al., 2008). An in vitro membrane insertion assay demonstrated that Hsp70 promoted the insertion of the viral replication proteins into the subcellular membranes (Wang et al., 2009). These functions of Hsp70 are not restricted to in vitro or yeast-based assays, since knockdown of cytosolic Hsp70 in plants or inhibition of Hsp70 with a chemical inhibitor were found to inhibit TBSV replication in a plant host (Wang et al., 2009).

The *SSA* subfamily of cytosolic Hsp70 consists of four genes and expression of at least one of the four *SSA* genes at high level is needed for yeast viability (Ahsen and Pfanner, 1997). It is currently unknown if the function of Ssa1/2p is essential for TBSV replication and whether additional cellular Hsp70 or other heat shock proteins can complement the functions of Ssa1/2p for supporting TBSV replication. To address these questions and to further dissect the roles of Hsp70 in TBSV replication, in this work, we used a yeast strain lacking functional *SSA2*, *SSA3* and *SSA4* genes, while the *SSA1* gene was either wt or temperature sensitive (ts). Based on the *ssa1*^{ts} strain, we demonstrate that the Ssa-subfamily of Hsp70 proteins is essential for TBSV replication in yeast. Using biochemical and temperature shift experiments, we show that Hsp70 is essential during the early steps of TBSV replication, but not during minus- and plus-strand synthesis.

Results

TBSV replication can be complemented by a heat shock-inducible host factor in ssa1ssa2 mutant yeast

Previous work has shown that *ssa1ssa2* mutant yeast can still support TBSV replication, albeit less efficiently and with significant delay when compared with the wt yeast (Wang et al., 2009). These observations indicate that either Ssa1p/Ssa2p are not essential for TBSV replication or they can be partially complemented possibly by other cellular chaperones. To test if complementation of TBSV replication can be enhanced by prior induction of heat shock proteins (Boorstein and Craig, 1990), we incubated *ssa1ssa2* mutant yeast at 42 °C for 30 min prior to the induction of p33/p92^{pol} proteins and the TBSV DI-72 repRNA. In comparison with *ssa1ssa2* mutant yeast grown at 23 °C all the time (no heat shock), which support only 15% repRNA accumulation, the *ssa1ssa2* mutant yeast treated with short heat-shock reached 80% repRNA accumulation of that measured in wt yeast (no heat shock) (Fig. 1A versus B). The stimulating effect of the short heat shock treatment prior to TBSV replication in *ssa1ssa2* mutant yeast suggests that a heat-inducible host factor, likely a heat-shock protein, can efficiently complement the missing Ssa1/2p functions for TBSV replication.

The purified heat-inducible Ssa3p Hsp70 can facilitate the assembly of the TBSV replicase in vitro

It is possible that the heat-inducible host factor supporting TBSV replication in *ssa1ssa2* mutant yeast, which could also be responsible for the partial complementation of TBSV replication at the standard temperature (Wang et al., 2009), is the cytosolic, stress-inducible Ssa3p and Ssa4p Hsp70 proteins, which are highly similar to one another and show 80% sequence identity with Ssa1/Ssa2p (Becker et al., 1996; Lin et al., 2001). Accordingly, Ssa3/4p are expressed at high levels in *ssa1ssa2* cells (Becker et al., 1996) and they can be efficiently induced by a short heat-shock treatment (Boorstein and Craig, 1990).

To study if Ssa3p could replace Ssa1p for supporting TBSV replication, we used the recently developed TBSV replication assay based on the addition of purified recombinant p33/p92^{pol} and the DI-72 repRNA to a yeast cell-free extract (Pogany et al., 2008). The in vitro assembled TBSV replicase is capable of supporting authentic TBSV repRNA replication if ribonucleotides are provided (Pogany and Nagy, 2008; Pogany et al., 2008). We have shown previously that Ssa1p is required for the in vitro assembly of the TBSV replicase when only the membrane fraction of the cell-free extract is used (Pogany et al., 2008).

To test if Ssa3p can support the assembly of the TBSV replicase in vitro, we used purified recombinant Ssa3p (fused with FLAG-tag) together with purified recombinant p33/p92^{pol} and the DI-72 repRNA and mixture of ribonucleotides in combination with the membrane fraction of the yeast cell-free extract. The in vitro replication assay