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Cellular Ubc2/Rad6 E2 ubiquitin-conjugating enzyme facilitates tombusvirus replication in yeast and plants

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ABSTRACT

Mono- and multi-ubiquitination alters the functions and subcellular localization of many cellular and viral proteins. Viruses can co-opt or actively manipulate the ubiquitin network to support viral processes or suppress innate immunity. Using yeast (*Saccharomyces cerevisiae*) model host, we show that the yeast Rad6p (radiation sensitive 6) E2 ubiquitin-conjugating enzyme and its plant ortholog, AtUbc2, interact with two tombusviral replication proteins and these E2 ubiquitin-conjugating enzymes could be co-purified with the tombusvirus replicase. We demonstrate that TBSV RNA replication and the mono- and bi-ubiquitination level of p33 is decreased in *rad6Δ* yeast. However, plasmid-based expression of AtUbc2p could complement both defects in *rad6Δ* yeast. Knockdown of *UBC2* expression in plants also decreases tombusvirus accumulation and reduces symptom severity, suggesting that Ubc2p is critical for virus replication in plants. We provide evidence that Rad6p is involved in promoting the subversion of Vps23p and Vps4p ESCRT proteins for viral replicase complex assembly.

Introduction

Plus-stranded (+)RNA viruses replicate in the infected cells by assembling membrane-bound viral replicase complexes (VRCs), which consist of viral- and host-coded proteins and the viral RNA template. Viral replication proteins of many (+)RNA viruses interact with various subcellular membranes that could result in the formation of spherules, which are single-membrane vesicles with a narrow opening towards the cytosol that harbor VRCs (de Castro et al., 2013; den Boon et al., 2010; Nagy and Pogany, 2012). Although major progress has recently been made in understanding of VRC assembly, the contribution of many host proteins to VRC assembly is far from complete (Belov and van Kuppeveld, 2012; Huang et al., 2012; Mine and Okuno, 2012; Nagy, 2008; Nagy and Pogany, 2008, 2012; Shulla and Randall, 2012).

Postranslation protein modification via addition of the highly conserved ubiquitin to substrate proteins by E1, E2 and E3 enzymes occurs frequently in eukaryotic cells (Popovic et al., 2014; van Wijk and Timmers, 2010). Poly-ubiquitination usually targets the substrate proteins for destruction by the 26 S proteasome. Alternatively, ubiquitination, especially mono- and multi-ubiquitination alters the biochemical properties and subcellular localization of substrate proteins (Popovic et al., 2014; van Wijk and Timmers, 2010). Ubiqutination also greatly affects the functions of many viral proteins and viruses actively manipulate the ubiquitin network to suppress innate immunity (Alcaide-Loridan and Jupin, 2012; Lindner, 2007; Okumura et al., 2006; Shackelford and Pagano, 2004; Shackelford and Pagano, 2005; Taylor and Barry, 2006).

The yeast Rad6p (radiation sensitive 6, also called Ubc2) E2 ubiquitin-conjugating enzyme is a member of the highly conserved UBC proteins in eukaryotes (Xu et al., 2009). Rad6p is involved in several cellular processes, including DNA repair, transcriptional activation and silencing, histone ubiquitination, ubiquitin-mediated N-end rule protein degradation and endoplasmic reticulum-associated protein degradation (ERAD) (Popovic et al., 2014; van Wijk and Timmers, 2010). Mutations in *RAD6* homologs are involved in many diseases (Popovic et al., 2014).

In *Arabidopsis*, 37 proteins with a UBC domain and active-site cysteine have been predicted. Among these *Arabidopsis* UBC proteins, the best-characterized *RAD6* homolog is *AtUBC2* (Xu et al., 2009). *AtUBC2* can partially rescue the UV-sensitivity and slow growth of *rad6Δ* yeast (Zwirn et al., 1997). AtUbc2p has E2 activity in vitro and has been shown to mono-ubiquitinate substrates in the absence of E3 ubiquitin ligases (Strzalka et al., 2013). *UBC2* is proposed to affect DNA repair, histone ubiquitination, flowering time, and enhance salt and drought-tolerance and

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modulate abiotic stress-induced gene expression in plants (Cao et al., 2008; Qin, 2013; Xu et al., 2009).

TBSV is a small (+)RNA virus that has been intensively used to study virus replication, recombination, and virus – host interactions based on yeast (*Saccharomyces cerevisiae*) model host (Nagy and Pogany, 2006, 2012; Nagy et al., 2014; Panavas and Nagy, 2003; Panaviene et al., 2004; White and Nagy, 2004). The auxiliary p33 replication protein, which is an RNA chaperone, recruits the TBSV (+)RNA to the cytosolic surface of peroxisomal membranes for replication (Jonczyk et al., 2007; McCartney et al., 2005; Nagy et al., 2012; Panavas et al., 2005a; Pogany et al., 2005; Stork et al., 2011). The interaction between the viral p33 and $p92^{pol}$ RdRp protein is required for assembling the functional VRC (Panavas et al., 2005a; Panaviene et al., 2004, 2005; Pogany and Nagy, 2008, 2012). The assembly and functions of VRCs are also affected by host components, such as the host heat shock protein 70 (Hsp70), the eukaryotic elongation factor 1 A (eEF1A), ESCRT (endosomal sorting complexes required for transport) proteins and sterols and phospholipids (Li et al., 2008; Li and Nagy, 2011; Li et al., 2009, 2010; Pogany and Nagy, 2012, 2015; Pogany et al., 2008; Serva and Nagy, 2006; Sharma et al., 2010, 2011; Wang et al., 2009a, 2009b; Xu and Nagy, 2015).

Genome-wide screens to identify host factors affecting TBSV RNA replication in yeast led to the identification of host genes known to be involved in various aspects of protein ubiquitination, such as *BRE1, DOA4, RAD6, LGE1, UBP3* (Jiang et al., 2006; Panavas et al., 2005b; Serviene et al., 2005, 2006). In addition, proteomics approach has revealed interaction between p33 replication protein and Uba1p ubiquitin- (Ub)-activating enzyme, Cdc34p E2 Ub-conjugating enzyme, Rsp5p E3 Ub-ligase, Ubp10p and Ubp15p Ub-specific proteases (Li et al., 2008). More detailed analysis with Cdc34p E2 Ub-conjugating enzyme showed that Cdc34p is present in the tombusvirus replicase complex and it can mono- and bi-ubiquitinate p33 in vitro in the absence of an E3 Ub-ligase (Li et al., 2008). The Nedd4-type Rsp5p E3 Ub-ligase has also been shown to bind to and ubiquitinylate p33 replication protein in vitro (Barajas et al., 2009b). Studies with the proteasomal Rpn11p metalloprotease, which acts as a deubiquitination (DUB) enzyme, has shown the role of Rpn11p in the assembly of TBSV VRCs, and the recruitment of the cellular DDX3-like Ded1p DEAD-box helicase into the viral replicase (Prasanth et al., 2014). Data also support the role of Rpn11p and the free ubiquitin pool in TBSV replication and viral RNA recombination (Prasanth et al., 2014). Altogether, the emerging idea from these studies on TBSV that ubiquitin and protein ubiquitination is a major element in virus replication and evolution.

Mono- and bi-ubiquitination of two lysines, namely K_{70} and $K_{76,}$ in a small fraction of p33 replication co-factor has been demonstrated in yeast (Barajas and Nagy, 2010; Li et al., 2008). Because mutations of these lysines reduced TBSV repRNA replication in yeast and affected the ability of p33 to interact with Vps23p ESCRT factor, we have proposed that one of the functions of p33 ubiquitination is to assist the recruitment of Vps23p ESCRT-I protein for TBSV replication (Barajas and Nagy, 2010). The recruitment of Vps23p, followed by subversion of additional ESCRT proteins could aid the formation of VRCs, which require membrane deformation to induce spherule-like structures (Barajas et al., 2009a, 2014).

Although the previous genome-wide and proteome-wide screens with TBSV in yeast have identified 10 yeast proteins involved in various aspects of the ubiquitin pathway (Nagy et al., 2014), we still do not know the functional roles of most of these cellular factors in virus replication. In the current paper, we have undertaken studies with Rad6p E2 ubiquitin-conjugating enzyme and its plant ortholog, *Arabidopsis thaliana* AtUbc2, in yeast and plants in combination with in vitro approaches. We find that both Rad6p and AtUbc2p interact with the p33 and $p92^{pol}$ replication proteins and they could be co-purified with the tombusvirus replicase. Rad6p/Ubc2p affects the ubiquitination level of p33 and deletion of *RAD6* or knockdown of *NbUBC2* reduces tombusvirus replication in yeast and plants, respectively. Both E2 ubiquitin-conjugating enzymes also facilitate TBSV replication in vitro, suggesting that they are directly involved in tombusvirus replication. We also provide evidence that Rad6p is involved in promoting the subversion of Vps23p and Vps4p ESCRT proteins for VRC assembly.

Results

The cellular Rad6/Ubc2 E2 ubiquitin-conjugating enzyme interacts with the tombusvirus p33 replication protein

To gain insights into the functions of Rad6/Ubc2 E2 ub-conjugating enzyme during tombusvirus replication, first we analyzed if p33 replication protein could interact with Rad6p or AtUbc2p. The membrane-based MYTH assay (split-ubiquitin-based yeast two-hybrid assay) between the tombusvirus p33 and the yeast Rad6p and p33:AtUbc2p revealed interactions (Fig. 1A). Moreover, interactions were also observed between p92:Rad6p and p92:AtUbc2p, respectively. (Fig. 1B).

In addition, FLAG-affinity-based co-purification experiments from the membrane fraction of yeast confirmed that Rad6p specifically interacted with FLAG-p33 (Fig. 1C, lane 2 versus 1). Also, the AtUbc2p expressed in yeast was co-purified with FLAG-p33 from the membrane fraction, suggesting interaction between p33 and AtUbc2p (Fig. 1C, lane 4 versus 3). Altogether, both Rad6p and AtUbc2p interacted with the p33 replication protein in the membrane and these E2 ubiquitin-conjugating enzymes might play a role during the assembly of the tombusvirus VRCs with a direct role in TBSV replication.

Deletion of RAD6 inhibits tombusvirus replication in yeast

To test if *RAD6* plays a pro-viral role in tombusvirus replication, we compared TBSV repRNA accumulation in *rad6Δ* and wt yeast co-expressing p33 and $p92^{pol}$ replication proteins. Northern blot analysis of total RNA from *rad6Δ* and wt yeasts revealed that the accumulation of TBSV repRNA was reduced by ~3-fold in *rad6Δ* yeast (Fig. 2A, lanes 6–10 versus 1–5). Thus, Rad6p likely plays a pro-viral function during tombusvirus replication.

Because Rad6p is an E2 ubiquitin-conjugating enzyme (Sung et al., 1991), it might affect the ubiquitination status of p33 replication protein, which has been shown to become mono- and bi-ubiquitylated in yeast (Barajas and Nagy, 2010). Accordingly, mono- and bi-ubiquinated forms of p33 were greatly reduced in *rad6Δ* yeast in comparison with wt yeast (Fig. 2B, lanes 5–6 versus 3–4). Thus, Rad6p is likely involved in ubiquitinylation of p33 replication protein in yeast.

One of the major functions of ubiquitination (i.e., poly-ubiquitination), is to tag proteins for degradation by the 26 S proteosome (Pickart, 2001; Pickart and Eddins, 2004). To test if deletion of *RAD6* affects the stability of p33, we tested the half-life of p33 molecules (Fig. 2C). After induction of expression of p33 from the *GAL1* promoter, we turned off p33 mRNA synthesis by changing the media from galactose to glucose, and addition of cycloheximide to block new protein translation, followed by taking samples at given time points. Levels of remaining p33 in yeast were measured by Western blot analysis (Fig. 2C). These experiments revealed that overall the half-life of p33 in *rad6Δ* yeast was comparable to that in wt yeast (Fig. 2C), suggesting that ubiquitination of p33 by Rad6p did not enhance p33 degradation.