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The Hop-Like Stress-Induced Protein 1 Cochaperone Is a Novel Cell-Intrinsic Restriction Factor for Mitochondrial Tombusvirus Replication

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ABSTRACT
Recent genome-wide screens reveal that the host cells express an arsenal of proteins that inhibit replication of plus-stranded RNA viruses by functioning as cell-intrinsic restriction factors of viral infections. One group of cell-intrinsic restriction factors against tombusviruses contains tetra/tricopeptide repeat (TPR) domains that directly interact with the viral replication proteins. In this paper, we find that the TPR domain-containing Hop-like stress-inducible protein 1 (Sti1p) cochaperone selectively inhibits the mitochondrial membrane-based replication of Carnation Italian ringspot tombusvirus (CIRV). In contrast, Sti1/Hop does not inhibit the peroxisome membrane-based replication of the closely related Tomato bushy stunt virus (TBSV) or Cucumber necrosis virus (CNV) in a yeast model or in plants. Deletion of STI1 in yeast leads to up to a 4-fold increase in CIRV replication, and knockdown of the orthologous Hop cochaperone in plants results in a 3-fold increase in CIRV accumulation. Overexpression of Sti1p derivatives in yeast reveals that the inhibitory function depends on the TPR1 domain known to interact with heat shock protein 70 (Hsp70), but not on the TPR2 domain interacting with Hsp90. In vitro CIRV replication studies based on isolated mitochondrial preparations and purified recombinant proteins has confirmed that Sti1p, similar to the TPR-containing Cyp40-like Cpr7p cyclophilin and the Ttc4 oncogene-like Cns1p cochaperone, is a strong inhibitor of CIRV replication. Sti1p interacts and colocalizes with the CIRV replication proteins in yeast. Our findings indicate that the TPR-containing Hop/Sti1 cochaperone could act as a cell-intrinsic virus restriction factor of the mitochondrial CIRV, but not against the peroxisomal tombusviruses in yeast and plants.

IMPORTANCE
The host cells express various cell-intrinsic restriction factors that inhibit the replication of plus-stranded RNA viruses. In this paper, the authors find that the Hop-like stress-inducible protein 1 (Sti1p) cochaperone selectively inhibits the mitochondrial membrane-based replication of Carnation Italian ringspot tombusvirus (CIRV) in yeast. Deletion of STI1 in yeast or knockdown of the orthologous Hop cochaperone in plants leads to increased CIRV replication. In addition, overexpression of Sti1p derivatives in yeast reveals that the inhibitory function depends on the TPR1 domain known to interact with heat shock protein 70 (Hsp70), but not on the TPR2 domain interacting with Hsp90. In vitro CIRV replication studies based on isolated mitochondrial preparations and purified recombinant proteins has confirmed that Sti1p is a strong inhibitor of CIRV replication. The authors’ findings reveal that the Hop/Sti1 cochaperone could act as a cell-intrinsic restriction factor against the mitochondrial CIRV, but not against the related peroxisomal tombusviruses.

Cells produce a yet-unknown number of cell-intrinsic restriction factors that limit replication of plus-stranded RNA [(+)
RNA] viruses. The cellular restriction factors could be virus specific or components of the cell-intrinsic innate systems of the host through targeting diverse pathogens (1–7). Cellular factors are also recruited by [(+)RNA viruses to aid viral replication, which takes place in membrane-bound viral replicase complexes (VRCs) in the cytoplasm of infected cells (8–16). The diverse, often opposite, roles of host factors are reflected by the identification of stimulatory as well as inhibitory host proteins in genome-wide screens with various hosts and viruses, such as Tomato bushy stunt virus (TBSV), West Nile virus, Brome mosaic virus (BMV), Hepatitis C virus (HCV), Dengue virus, and Drosophila virus C (17–25). However, the detailed functions of the majority of the identified host proteins in [(+)RNA virus replication have not been fully revealed.

TBSV is a plant-infecting [(+)RNA virus used extensively to study virus replication, recombination, and virus-host interac-

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cochaperone are strong inhibitors of TBSV replication in yeast and in vitro (41, 42). Additional cellular chaperonins, such as the CypA, and the related Ess1p parvulin also decrease TBSV RNA accumulation in yeast and plants (36, 41, 43). Moreover, the cellular nucleolin, an RNA-binding protein, inhibits TBSV replication by blocking the recruitment of the viral RNA into replication (44). Another group of cellular restriction factors is the WW motif-containing host proteins, such as Rsp5p Nedd4-like E3 ubiquitin ligase, which regulate the degradation of tombusviral p92pol in yeast cells and inhibit the activity of VRC in vitro (45, 46).

Cellular kinases, such as Pck1p, could also restrict TBSV replication in yeast (32). Taken altogether, studies of cellular restriction factors could help to unravel the full arsenal of the native cell-intrinsic innate immune system in the host cell.

Similar to other (+)RNA viruses, tombusviruses, such as TBSV, use intracellular membranes for replication. Interestingly, TBSV utilizes the peroxisomal membrane, while the closely related Carnation Italian ringspot virus (CIRV) takes advantage of the outer mitochondrial membranes to build VRCs in infected plants and yeast (47–49). The two viral replication proteins (i.e., p33 and p92pol for TBSV and p36 and p95pol in the case of CIRV) is known to coopt 8 to 10 host proteins to assemble the tombusvirus VRC (37–39, 50–52). The highly homologous p33 of TBSV and p36 of CIRV replication proteins are master regulators of replication, playing a multifunctional role in recruitment of the tombusviral (+)RNA to the site of replication, the assembly of the VRC, and viral RNA synthesis by acting as RNA chaperones (50, 53–57). The RdRp protein p92pol of TBSV and p95pol of CIRV are also components of the functional VRCs (28, 55, 57–59). The subverted host proteins have been shown to bind to the viral RNA and the viral replication proteins (8, 39, 60). Detailed studies showed that heat shock protein 70 (Hsp70), euakaryotic elongation factor 1A (eEF1A), and several members of the endosomal sorting complexes required for transport (ESCRT) family of host proteins are required for the assembly of VRCs (52, 61–64). Additional subverted host proteins include the DDX3-like Ded1p/AtRH20 and the human p68-like Dbp2, the eIF4AIII-like Fall/AtRH2 and DDX5-like Dbp3/AtRH5 DEAD-box RNA helicases, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), eEF1Bγ, and eEF1A, all of which have been shown to affect viral RNA synthesis (51, 60, 61, 65–68).

Previous works with TBSV revealed the unexpected inhibitory function for several tetraticopeptide repeat (TPR) domain-containing proteins, such as the CypA40-like Cpr7p cyclophilin and Ttc4-like Cns1p cochaperone in yeast and in vitro (41, 42). Mechanistic studies showed that the inhibitory effect of Cpr7p was due to its interaction with the RNA-binding domain of the tombusviral p33 replication protein that leads to inhibition of p33/p92pol-based recruitment of the TBSV (+)RNA for replication and a decrease of the efficiency of the VRC assembly. Importantly, the key element in Cpr7p was not the cyclophilin domain, but its TPR domain consisting of three TPR modules in Cpr7p (41). Similarly, via its TPR domain, Cns1p bound to the tombusviral p33 and p92pol replication proteins and inhibited VRC assembly and reduced TBSV replication in yeast and in vitro based on a yeast cell-free extract (CFE) assay (42). However, in case of Cns1p, the interaction targeted the p33-p33/p92 interaction domain, suggesting that TPR-containing cellular proteins might restrict TBSV replication via different mechanisms.

The TPR domain consists of repeats of a 34-amino-acid sequence adopting a right-handed helical loop-helix structure with an amphipathic channel; such channels are involved in many protein-protein interactions (69, 70). Although the TPR domains are highly variable, which likely affects substrate specificity, the canonical TPR domain contains a pattern of small and large hydrophobic amino acids. The TPR domain proteins are abundant in all kingdoms of life, including 246 proteins in Arabidopsis, 338 in mouse, 63 in Caenorhabditis elegans and 26 in yeast (Interpro database, http://www.ebi.ac.uk/interpro/entry/IPR013026/taxonomy) (71). TPR domain proteins function in protein trafficking, protein import to organelles, transposon silencing, apoptosis, and synaptic vesicle fusion (72, 73). Various TPR domain proteins are involved in numerous human diseases, such as cancer, amyloidosis, cystic fibrosis, prion protein propagation, and bacterial pathogenesis (74–79). Several TPR domain proteins have been shown to affect infections by viruses, such as Chikungunya virus, West Nile virus, Vesicular stomatitis virus, Herpes simplex virus, Poxvirus, and baculoviruses (80–85). TPR domain proteins are also important in interferon-induced antiviral responses, including the IFN-induced protein with tetraticopeptide repeat (IFIT) protein family (5, 85–88).

Our previous discoveries invited our attention to TPR-like sequences, including the well-studied stress-induced protein 1 (Sti1p in yeast, Hop protein in mammals and plants) cochaperone. Sti1p, which is a conserved highly abundant protein lacking chaperone activity on its own, is a cochaperone of Hsp70 and Hsp90 chaperones (89, 90). Sti1p contains three TPR domains, which are involved in binding to Hsp90s and Hsp70s. Sti1p plays a role in client protein transfer from the Hsp70 complex to the Hsp90 complex. Interestingly, Sti1p can simultaneously bind to Hsp70 and Hsp90, and by inhibiting the ATPase activity of Hsp90, Sti1p stabilizes the ternary Hsp70-Hsp90-client protein intermediate complex (91, 92).

In this paper, we show that the yeast Sti1p cochaperone has a strong inhibitory function during the mitochondrial CIRV replication but not in the peroxisomal tombusvirus replication. Detailed analysis of Sti1p revealed that it interacted with the RNA-binding domain of CIRV p36 replication protein and ultimately restricted VRC assembly in vitro and CIRV RNA accumulation in yeast and the orthologous Hop inhibited CIRV accumulation in plants. Thus, TPR-containing cellular cochaperone proteins emerge as new cell-intrinsic restriction factors of a mitochondrial (+)RNA virus.

MATERIALS AND METHODS

Yeast strains and expression plasmids. Yeast strains BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and sti1Δ (single-gene deletion strain) were obtained from Open Biosystems (Huntsville, AL, USA). For tombusviral replication in yeast, pESC-HisCNPv3p-D172, pYES-CNPv92, pESC-C36/D172, and pYES-C95 were described previously (57). To generate pESC-C36/D1, CIRV DI-1 (93) (constructed by D. Barajas and P. D. Nagy, unpublished data) was PCR amplified using primer pair 4124 (CCGGAATTCAGAAATATCTCAGGATTTGACCGTCC)/1069 (CCGG TCGAGCTTACAGGTAATATACCCACAACTGTTG), digested with EcoRI/Sacl, and then inserted into EcoRI/Sacl-digested pESC-HisCNPv33p-D172, generating pESC-HisCNPv33-D1. Cucumber necrosis virus (CNV) p33 sequence was then removed by BamiHI/Xhol digestion and replaced with BamiHI/Xhol-digested CIRV p36 sequence, which was PCR amplified from pESC-C36/D172 using primer pair 900 (CGACGGATCC GAGGTTGAAGCGCTAATGGTG)/3230 (CCGGCTGAGCTATT TGACACGGAGGATT), generating pESC-C36/D1.
To generate Twin-Strep-tagged CIRV and CNV replication proteins, the primer pairs 5351 (CATCCACATTGAAAAATCTGGTGGGA GGTTCCTGATCATGACATCAACGATGAGTGA/952) (CCCGCGTCTG CTACATGCAGGGCCGCAGAAGGA), 5350 (GTGGTTGCTGGTT GGTTGGTTGCTGGTTGCTCTACCATACAAAATCCTAAGG/952), and 5349 (GGAGACTAACTTAAATGTTTTTCTGTTGCACCTACA TCCAAATTCGAAAAAGGTGGTGGTTGCTGGTTGCTGGT GG)/952 were sequentially inserted into pESC-DI72, generating pESC-StrepCNVp92/DI72.

CIRV p95 sequence was amplified using primer pair 900/3230 and inserted into pESC-DI72, generating pESC-StrepCNVp92/DI72, as described previously (97). Replication was calculated by measuring the accumulation of CIRV DI1 repRNA or the TBSV DI-72 (+) repRNA relative to the accumulation of 18S rRNA. The tombusvirus replication protein analysis was performed as described previously using an anti-His6 antibody as the primary antibody for the detection of His6-p36 and His6-p95. Detection of Flag-Sti1p and Sti1p was carried out using primary anti-Flag and anti-Sti1 antibody, respectively. The secondary antibody for both primary antibodies was alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Sigma) (41).

Analysis of protein–protein interaction by split-ubiquitin assay. The bait constructs, pGAD-BT2-N-His36 and pGAD-BT2-N-His33, expressing CIRV replication protein p36 and p33 tombusvirus replication protein, have been published before (36, 38). The PCR products of the STI1 gene and its various truncation versions were digested with BamHI/HindIII and ligated into the pPRN-N-RE vector digested with BamHI/Sall enzymes. The PCR products of AtHop-1 (96) was digested with BclI and ligated into the pPRN-N-RE vector digested with BclI/Sti1 enzymes. Yeast strain sti1Δ/NMY51 was cotransformed with pGAD-BT2-N-His36 or pGAD-BT2-N-His33 and pPR-N-RE (NubG) or one of the prey constructs carrying the STI1 gene and plated onto Trp-/Leu-/(TL-) synthetic minimal medium plates for plasmid selection (36, 38). Yeast colonies were resuspended in 50 μl water and spotted onto Trp-/Leu-/His-/Ade- (TLHA-) plates for 2 to 4 days to detect bait-prey interactions. Plasmid containing the yeast SSA1 Hsp70 gene served as the positive control and an empty vector (pPR-N-RE) as the negative control in this assay (36, 38).

Protein purification from E. coli. pMAL-p33 (TBSV p33), pMAL-p92 (TBSV p92), pMAL-p36 (CIRV p36), and pMAL-p95 (CIRV p95) (40) were transformed separately into E. coli strain BL21 (DE3) CodonPlus. Protein expression was induced using isopropyl β-D-thiogalactopyranoside (IPTG) for 8 h at 16°C, and the cells were harvested by centrifugation at 5,000 rpm at 4°C for 5 min to remove the medium prior to –80°C storage. Affinity columns containing amyllose resin (NEB) were used to purify maltose-binding protein (MBP)-tagged recombinant proteins. The frozen pellets were suspended and sonicated in MBP column buffer containing 20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The sonicated extract was centrifuged at 15,000 rpm, and the supernatant was added to the preequilibrated amyllose resin for 1-h rotating incubation at 4°C. After the resin was washed three times with column buffer and once with a low-salt column buffer (25 mM NaCl), the proteins were eluted with a low-salt column buffer containing 0.18% (vol/wt) maltose and stored at –80°C in 6% (vol/vol) glycerol. Protein fractions used for the replication assays were 95% pure, as determined by 12% SDS-PAGE and staining with Coomassie blue.

Expression of glutathione S-transferase (GST)–tagged proteins Cpr7p, Cpr7-TPR, Cpr7-NF, and the mutated versions (C49Y, G325D, ΔTPR1, and ΔTPR2) were induced using IPTG for 6 h at 23°C, and the cells were harvested by centrifugation at 5,000 rpm at 4°C for 5 min to remove the medium and stored at –80°C. Purification of GST–tagged proteins was carried out using glutathione resin and eluted with 10 mM N-Acetyl-L-cysteine (NAC). The protein purification was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
glutathione–10 mM β-mercaptoethanol in the column buffer following the same protocol as that for MBPs.

In vitro tombusvirus replication assay using yeast mitochondrial preparations. Yeast intact mitochondria were purified as described previously (40). The purified mitochondrial fraction (1 μl) and different dilutions of GST, Sti1p, Cpr7p, or Cns1p proteins (8, 16, and 32 μM each) were incubated at 25°C for 1 h in 8 μl buffer A (containing 30 mM HEPES-KOH [pH 7.4], 150 mM potassium acetate, 5 mM magnesium acetate, and 0.6 M sorbitol) with 15 mM creatine phosphate, 1 mM ATP, and GTP, 0.1 mg/ml creatine kinase, 0.1 μl of RNase inhibitor, 10 mM dithiothreitol, 0.5 μM DI-72 RNA transcript, and affinity-purified 0.5 μg MBB-36 (CIRV p36) and MBP-p95 (CIRV p95). The volume of the reaction mixture was then adjusted by adding 16 μl buffer B (containing 30 mM HEPES-KOH [pH 7.4], 150 mM potassium acetate, and 5 mM magnesium acetate) with 15 mM creatine phosphate, 1 mM ATP, CTP, and GTP, 0.025 mM UTP, 0.2 μl of [32P]UTP, 0.1 mg/ml creatine kinase, 0.2 μl of RNase inhibitor, 10 mM dithiothreitol, and 0.05 mg/ml actinomycin D. The reaction mixture was incubated at 25°C for 3 h and terminated by adding 100 μl stop buffer [1% sodium dodecyl sulfate and 0.05 mM EDTA [pH 8.0]] followed by 100 μl phenol-chloroform extraction and isopropanol-ammonium acetate precipitation overnight at −20°C and washing by 70% ethanol. The newly synthesized 32P-labeled RNA products were incubated at 85°C for 5 min and separated by electrophoresis in a 5% polyacrylamide gel containing 0.5× Tris-borate-EDTA buffer with 8 M urea. Signals were detected using a Typhoon 9400 imaging scanner (GE-Amersham) and quantified by ImageQuant software.

Copurification of host proteins with Twin-Strep-tagged CIRV replication proteins from yeast. To purify the protein of interest, 200 mg of BY4741 yeast cells were transformed with plasmids pESC-Strep-C36/DI72 pYES-StrepC95 and pRS315-Sti1p. Cultured yeasts were resuspended and homogenized in buffer B (50 mM Tris-HCl [pH 7.5], 15 mM MgCl2, 10 mM KCl, 10 mM β-mercaptoethanol, 1% [vol/vol] yeast protease inhibitor cocktail) by glass beads (modified from reference 28). Membrane fractions from cell homogenates were collected and solubilized with column buffer (50 mM Tris-HCl [pH 7.5], 15 mM MgCl2, 500 mM KCl, 1% Triton X-100, 5% caprylyl sulfobetaine [SB3-10; Sigma]), 10 mM β-mercaptoethanol, 1% [vol/vol] yeast protease inhibitor cocktail), and incubated with 40 μl Strep-Tactin Superflow high-capacity 50% resin (IBA Life Sciences) for 1 h at 4°C in a column. Strep-Tactin resin was then washed twice with column buffer, two times with wash buffer (50 mM Tris-HCl [pH 7.5], 15 mM MgCl2, 10 mM KCl, 0.1% Triton X-100, 10 mM β-mercaptoethanol, 1% [vol/vol] yeast protease inhibitor cocktail), and eluted with SDS-PAGE loading buffer and then subjected to SDS-PAGE and Western blotting with Strep-Tactin AP conjugate (IBA Life Sciences) and anti-Flag and anti-Hsp70 antibodies (Abcam).

Confocal laser microscopy. Wild-type (wt) BY4741 or sti1Δ yeast strains were transformed with the following expression plasmids: pESC-GFP-C36/DI72 pYES-C95, pESC-GFP-C33/DI72 pYES-C92 (57), as well as pRS315-RFP-Sti1p. The yeast cultures were incubated in galactose medium overnight, sampled, and imaged with an Olympus FV1000 confocal laser scanning microscope (Olympus America Inc., Melville, NY). The microscope settings were the following: excitation and emission for 488-nm laser/500- to 530-nm filter and 543-nm laser/560- to 660-nm filter. Yeast-based studies reveal that Sti1p cochaperone selectively inhibits mitochondrial CIRV replication but not the peroxisomal TBSV replication. Based on our previous findings that two abundant cytosolic TPR-containing cellular proteins, namely, Cpr7p cyclophilin and Cns1p cochaperone, showed robust restriction activity against TBSV (41, 42), we also tested the abundant TPR-containing protein Sti1p cochaperone for possible effects on the accumulation of TBSV and CIRV replicon RNAs (repRNAs) in sti1Δ yeast versus wt yeast cells. Interestingly, sti1Δ yeast supported CIRV repRNA accumulation at an ~3-fold higher level than that for wt yeast (Fig. 1A, lanes 5 and 6 versus lanes 1 and 2). Replication of the TBSV repRNA was comparable in sti1Δ and wt yeast (Fig. 1B, lanes 4 to 6 versus lanes 1 to 3), suggesting that Sti1p has a CIRV-specific inhibitory effect. To test if Sti1p-based inhibition targets the CIRV RNA, we also tested TBSV repRNA accumulation in the presence of CIRV p36 and p95pol replication proteins, which are capable of supporting the replication of the heterologous TBSV repRNA (57), in sti1Δ yeast versus wt yeast cells. The obtained data showed a ~2-fold increased level of TBSV repRNA accumulation (Fig. 1C, lanes 4 to 6 versus lanes 1 to 3), demonstrating that the inhibitory effect of Sti1p is targeted against CIRV p36 and p95pol and not the viral RNA. Comparison of the accumulation of CIRV p36 and p95pol in sti1Δ yeast versus wt yeast cells revealed similar replication protein levels (Fig. 1C), arguing that Sti1p is unlikely to affect translation or stability of CIRV p36 and p95pol in yeast cells.

To further test if Sti1p can inhibit CIRV replication in vivo, we overexpressed N-terminally FLAG-tagged Sti1p in yeast supporting CIRV or TBSV accumulation. We found that overexpression of Sti1p reduced CIRV accumulation up to ~3-fold in wt yeast (Fig. 2A, lanes 4 to 6 versus lanes 1 to 3) and ~5-fold in sti1Δ yeast (Fig. 2A, lanes 10 to 12 versus lanes 7 to 9). The level of CIRV p36 and p95pol replication proteins was comparable in sti1Δ and wt yeasts overexpressing FLAG-Sti1p (Fig. 2A), suggesting that Sti1p is unlikely to affect the stability of these viral proteins in yeast. In contrast, replication of the TBSV repRNA was not affected by the overexpression of Sti1p in wt (Fig. 2B, lanes 13 to 16) or sti1Δ (lanes 17 to 24) yeasts. Altogether, these data support the idea that Sti1p is a strong inhibitor of the CIRV p36 and p95pol replication proteins, while Sti1p seems to be ineffective against the tombusviral p33 and p92pol replication proteins in yeast.

The binding of Sti1p involves different regions in CIRV p36 and the TBSV p33 replication proteins. Sti1p contains three TPR domains (Fig. 3A) that are predicted to interact with the tombusviral replication proteins. To test if Sti1p can interact with the
CIRV p36 versus the TBSV p33 replication proteins, we first used the split-ubiquitin-based two-hybrid assay with *sti1Δ* yeast (99, 100). We observed a strong interaction between Sti1p and p36 (Fig. 3B) and Sti1p and p33 (Fig. 3C). We confirmed the interaction between Sti1p and p36 replication protein in *sti1Δ* yeast (Fig. 3D, lane 2) using a copurification assay with recombinant Sti1p. The reciprocal copurification assay with Strep-tagged p36 also resulted in copurification of Flag-Sti1p from *sti1Δ* yeast (Fig. 3E, lane 1).

To test what region(s) of Sti1p interacts with p36, we used well-characterized Sti1p mutants lacking particular functional domains (94, 95) as shown in Fig. 3A. The split-ubiquitin assay showed that the interaction with p36 was not eliminated by deletion (ΔTPR1) or mutation (C9V and K73E) in the TPR1 region (Fig. 3B), which binds to Hsp70 (89, 101). Similarly, deletion (ΔTPR2) or mutation (G325D or T526I) in the TPR2 region (Fig. 3B), which binds to Hsp90, did not debilitate interaction with p36 replication protein. These findings were confirmed in the recipro-

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**FIG 1** Increased CIRV replication in *sti1Δ* yeast. (A) Northern blot analysis of accumulation of CIRV DI-1 repRNA in *sti1Δ* or wt yeast strains at 23°C. We launched CIRV repRNA replication by expressing CIRV His6-p36 and His6-p95 from the galactose-inducible *GAL1* promoter and DI-1 (+) repRNA from the galactose-inducible *GAL10* promoter in *sti1Δ* and the parental (wt; BY4741) yeast strains. We also overproduced the FLAG-tagged Sti1p in wt yeast to test its inhibitory function. Note that the data were normalized based on 18S rRNA. Each experiment was repeated three times. (B) Northern blot analysis of accumulation of TBSV DI-72 repRNA in *sti1Δ* or wt yeast strains. TBSV repRNA replication was launched by expressing CNV His6-p33 and CNV His6-p92 from the *GAL1* promoter and DI-72 (+) repRNA from the *GAL10* promoter in *sti1Δ* and the parental (wt; BY4741) yeast strains. See further details in panel A. Bottom images, Western blot analysis of CNV His6-p33, CNV His6-p92 accumulation by anti-His antibody, and Sti1p accumulation by anti-Sti1 antibody. (C) Top images, Northern blot analysis of the CIRV p36/p95-driven TBSV DI-72 RNA accumulation in *sti1Δ* or wt yeast strains. Same as panel A except DI-72 was used as a repRNA with CIRV His6-p36 and His6-p95, which support viral RNA replication on mitochondrial membrane surfaces. Bottom images, Western blot analysis of CIRV His6-p36, CIRV His6-p95 accumulation by anti-His antibody, and Sti1p accumulation by anti-Sti1 antibody. Each experiment was repeated three times.
cal copurification experiments (Fig. 3D and E), demonstrating that Sti1p could use both the TPR1 and TPR2 sequences to bind to the p36 replication protein. Interestingly, binding of Sti1p to the TBSV p33 replication protein showed features similar to those of p36 binding (Fig. 3C versus Fig. 3B). Thus, the binding characteristics of Sti1p to CIRV p36 versus TBSV p33 do not explain why Sti1p can selectively inhibit CIRV replication, but not TBSV replication, in yeast cells.

To map the Sti1p-binding site in the CIRV p36 replication protein, we have used pulldown experiments with immobilized MBP-p36 truncation derivatives (Fig. 4A) and Sti1p present in either E. coli lysate (Fig. 4B) or yeast extract containing Flag-Sti1p (Fig. 4C). These experiments revealed that Sti1p binds to a region that includes the arginine- and proline-rich (RPR) motif of p36, which is involved in RNA binding (Fig. 4C versus Fig. 3B). Thus, the binding characteristics of Sti1p to CIRV p36 versus TBSV p33 do not explain why Sti1p can selectively inhibit CIRV replication, but not TBSV replication, in yeast cells.

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Sti1p is colocalized with CIRV p36 in yeast cells. To study if the mostly cytosolic Sti1p is recruited to the mitochondrial membranes, where CIRV replication takes place (49, 57), by the CIRV p36, we coexpressed GFP-p36 with RFP-Sti1p in wt or sti1Δ yeast cells. Confocal laser microscopy revealed the robust recruitment of RFP-Sti1p by CIRV the RPR-containing sequence) and TBSV replication proteins (the C-terminal region in TBSV p33), suggesting that the mechanism of inhibition of CIRV replication by Sti1p could be based on blocking the RNA-binding function of CIRV p36 replication protein.

Xu et al.

9366 jvi.asm.org Journal of Virology

FIG 2 Overexpression of Sti1p inhibits CIRV accumulation in yeast. (A) Top panel, Northern blot analysis of CIRV RNA accumulation in wt or sti1Δ yeasts overproducing the FLAG-tagged Sti1p. Second panel, Northern blot analysis to demonstrate the comparable level of rRNA loading in the yeast samples. Bottom panels, Western blot analysis of CIRV His6-p95 and CIRV His6-p36 accumulation by anti-His antibody and Sti1p accumulation by anti-Sti1 antibody. (B) Northern blot analysis of TBSV DI-72 repRNA accumulation in wt or sti1Δ yeasts overproducing the FLAG-tagged Sti1p in the presence of peroxisomal CNV p33/p92 replication proteins. Each experiment was repeated three times. See further details in legend to panel A.

FIG 2 Overexpression of Sti1p inhibits CIRV accumulation in yeast.
RFP-Sti1p showed a diffused, mostly cytosolic distribution in yeast expressing p33 replication protein or in the absence of viral proteins (Fig. 5C). Based on these data, we suggest that, unlike p33, the CIRV p36 replication protein efficiently recruits Sti1p to the site of replication, leading to robust inhibition of CIRV replication.

The TPR1 domain in Sti1p is required to inhibit CIRV replication in isolated mitochondrion-based assay and in yeast cells. To test what domain of Sti1p is required to block CIRV replication, we expressed mutated versions of Sti1p in wt yeast. We observed a 2-fold inhibition of CIRV repRNA accumulation by TPR1 and G325D mutants, comparable to that obtained with the full-length Sti1p (Fig. 6A), while expression of ΔTPR1 or C49Y mutants had no detectable and lesser inhibitory effects, respectively (lanes 9 to 12 and 21 to 24 in Fig. 6A). All these mutated versions of Sti1p were expressed at comparable levels in wt yeast without substantially affecting CIRV p36 or p95 levels (Fig. 6B and C). Altogether, based on these data, we suggest that the TPR1 domain of Sti1p is required to inhibit CIRV replication in yeast.

To further test the roles of the TPR sequences of Sti1p in CIRV replication, we applied an isolated mitochondrion-based replication assay, which takes advantage of purified recombinant CIRV p36 and p95 repRNA transcripts to support full CIRV replication in vitro (Fig. 7A) (57, 58, 104). Addition of the affinity-purified full-length recombinant GST-Sti1p (Fig. 7B) decreased the production of repRNA up to 3-fold (Fig. 7C, lanes 5 to 7 versus lanes 2 to 4), confirming that Sti1p has an inhibitory effect on CIRV replication in vitro. Preincubation of

FIG 3 Interaction between Sti1p and CIRV p36 replication protein in yeast and in vitro. (A) Domain structure of the yeast Sti1p. Tetratricopeptide repeat 1 (TPR1) sequence interacts with Hsp70, while dipeptide repeat of aspartic acid and proline 1 (DP1) might stabilize the bound client protein. TPR2A and TPR2B bind to Hsp90 and together inhibit the ATPase activity of Hsp90. TPR2B also binds to Hsp70, but only in concert with Hsp90 binding to TPR2A. The debilitating mutations are marked with an asterisk, and deletion constructs are shown schematically at the bottom of the panel. (B) A split‐ubiquitin MYTH assay was used to test intracellular interaction between CIRV p36 and the wt or mutated yeast Sti1p. The bait p36 was coexpressed with the prey Sti1p protein in sti1Δ yeast. The empty prey vector (NubG) was used as positive and negative controls, respectively. (C) Same split‐ubiquitin MYTH assay as that shown in panel B, except that TBSV p33 was used as a bait protein. (D) Copurification of CIRV p36 replication protein with the yeast Sti1p from yeast cells. The membrane fraction of yeast coexpressing the wt or mutated FLAG-Sti1p and His6-p36 was solubilized, and the Sti1p variants were purified using a FLAG column. The eluted proteins were tested using Western blotting with anti-FLAG antibody (top image) and anti-His antibody (bottom image). (E) Reciprocal copurification of the yeast Sti1p with CIRV p36 and p95 replication proteins from yeast cells. Details are as described for panel D, except yeast coexpressed the Twin-Strep-tagged CIRV p36 and p95 and Flag-Sti1p. The purification was based on Strep-Tactin columns. The eluted proteins were tested using Western blotting with anti-Strep-Tactin-AP conjugate (top image) and anti-Flag antibody (middle image), and anti-Hsp70 antibody (bottom image). Note that the coopted Hsp70 is a permanent member of the tombusvirus replicase complex. Each experiment was repeated three times.

(55, 105).
Sti1p either with Ssa1p Hsp70 chaperone or p36 replication protein to facilitate protein complex formation did not alter the inhibitory effect of Sti1p (Fig. 7D), suggesting that Sti1p has a robust effect on CIRV replication in vitro. The presence of recombinant Sti1p lacking a functional TPR2 domain (e.g., ΔTPR2 or mutant G123D) was also inhibitory, reducing CIRV replication up to ~5-fold in the isolated mitochondrion-based assay (Fig. 7C, lanes 11 to 16, and 7D, lanes 15 to 22). In contrast, inactivation of TPR1 (ΔTPR1 or mutant C49Y) resulted in the loss of the inhibitory function of Sti1p (Fig. 7C, lanes 8 to 10 and 17 to 19, and 7D, lanes 11 to 14) in the isolated mitochondrion-based replication assay, thus emphasizing the critical role of the TPR1 sequence in Sti1p.

Comparison of the inhibitory effects of host proteins carrying TPR domains on CIRV replication in isolated mitochondrion-based replication assay. Two other cellular proteins with TPR domains, namely, Cpr7p cyclophilin and Cns1p cochaperone, have been shown to inhibit TBSV replication (41, 42). To test if these host proteins have activities comparable to those of Sti1p in the inhibition of CIRV replication, we used the isolated mitochondrion-based in vitro replication assay and purified recombinant cellular proteins and CIRV replication proteins (Fig. 8A and B). Interestingly, all these TPR-containing proteins inhibited CIRV replication in vitro with Sti1p and the TPR region of Cpr7p, showing up to an ~10-fold reduction in repRNA production in the isolated mitochondrion-based replication assay (Fig. 8A, lanes 6 to 8 and 12 to 14 versus lanes 3 to 5). Cns1p was the least effective in this assay (Fig. 8A, lanes 15 to 17), but this could be due to the smaller amount of recombinant GST-Cns1p obtained from E. coli (Fig. 8B). However, the purified recombinant GST-Cns1p was the most effective inhibitor of TBSV replication, reducing TBSV replication up to ~20-fold in a cell-free extract (CFE)-based replication assay (Fig. 8C) (42). Altogether, the TPR-containing Cpr7p seems to have a strong inhibitory effect against both TBSV and CIRV replication, while Sti1p efficiently inhibits CIRV, but its effect on TBSV replication in vitro is only moderate.

The plant Hop ortholog of the yeast Sti1p inhibits CIRV replication in yeast and plants. Arabidopsis thaliana has three orthologs of Sti1p cochaperone, namely, AtHop1-3, that carry TPR1 and TPR2 domains (96, 106). AtHop-1 has 558 amino acids that show 40% identity and 57% similarity with ScSti1, which is 589 amino acids long. Using the membrane yeast two-hybrid (MYTH) assay, we showed a strong interaction between CIRV p36 replication protein and the AtHop-1 ortholog (Fig. 9A). These data confirmed the pulldown experiments with recombinant AtHop-1 and CIRV p36 that also showed the involvement of the RPR domain in p36 in the interaction with AtHop-1 (Fig. 4D).

To test if AtHop-1 could inhibit CIRV replication, we expressed it in yeast. Similar to results with the yeast Sti1p, AtHop-1 inhibited CIRV accumulation ~5-fold in yeast (Fig. 9B, lanes 4 to 6). Deletion of the TPR1 sequence made AtHop-1 less effective.
inhibitor of CIRV replication (Fig. 9B, lanes 7 to 9), suggesting that the TPR1 sequence is important for the inhibitory function of AtHop-1.

To test the relevance of the plant Hop protein in tombusvirus replication, we tested the accumulation level of Hop mRNA in the Nicotiana benthamiana host. The semiquantitative reverse transcription-PCR (RT-PCR) analysis revealed the induction of NbHop mRNA upon infection with CIRV (Fig. 10A). In addition, knockdown of NbHop level via VIGS in N. benthamiana led to 3-fold-increased CIRV genomic RNA accumulation (Fig. 10B). As expected, due to the high level of CIRV accumulation, the Hop-knockdown N. benthamiana plants developed more severe symptoms than control plants when infected with CIRV (Fig. 10C). In contrast, the accumulation of the genomic RNA of the related CNV (a peroxisomal replicating tombusvirus, closely related to TBSV) was not significantly affected by Hop-knockdown N. benthamiana plants (Fig. 10D). Based on these in planta experiments, we suggest that the plant Hop ortholog plays a potent inhibitory role, similar to that of the yeast Sti1p, in the mitochondrial CIRV replication, but not in the peroxisomal CNV replication.

DISCUSSION
Identification of the Hop-like Sti1p co-chaperone as a novel cell-intrinsic restriction factor against CIRV replication in mitochondria. Cellular protein chaperones are important for virus replication and during other steps of the infectious process (10, 107–113). For example, Hsp70 has been shown to affect the intracellular localization and membrane insertion of TBSV replication proteins and the assembly of the tombusviral VRCs (62, 63, 104). Although Hsp70 interacts directly with the tombusviral replication proteins, it is possible that other cellular factors could affect the subversion of Hsp70s by TBSV. Since cochaperones facilitate selection and delivery of client proteins to the major Hsp70 and Hsp90 chaperones (114–116), some cochaperones might also be involved in viral infections, as demonstrated in this paper and earlier (10, 107–113).

Our finding that the conserved cellular Hop-like Sti1p co-chaperone is a restriction factor for CIRV replication in the mitochondria contributes to the emerging complex roles of cellular chaperones in virus replication (10). While deletion of Sti1p led to a 2- to 4-fold increase in CIRV replication in the yeast model host, and knockdown of the orthologous Hop in N. benthamiana increased CIRV accumulation ~3-fold, overexpression of Sti1 or AtHop-1 in yeast was inhibitory. In vitro CIRV replication experiments based on isolated mitochondria also confirmed the robust inhibitory effect of Sti1p on CIRV. Moreover, the expression of the Sti1 ortholog Hop is increased during CIRV replication in plant leaves. Thus, Sti1p is a new member of the growing family of cell-intrinsic restriction factors.

However, Sti1p did not have a robust effect on replication of the closely related TBSV in yeast or on the replication of CNV in plants, both of which utilize the peroxisomal membranes for replication (55, 105). This contrasting finding with different tombusviruses exploiting different subcellular locations could be due to the difference in accessibility of Sti1/Hop to replication proteins of tombusviruses in their respective cellular environments. For example, it has been shown in plants that Hop/Sti1 is involved in transportation of freshly synthesized mitochondrial and chloroplast proteins from the cytosol into these organelles (117). Moreover, the delivery/import of mitochondrial preproteins from the cytosol to the mitochondria often depends on Hsp70/Hsp90 chaperones and includes Hop/Sti1 and the TPR domain in Tom70.
mitochondrial receptor (118, 119). Also, the CIRV p36 replication protein was shown to interact with various Tom receptor proteins, which might have roles in mitochondrial membrane insertion of p36 (47). Based on these studies, we propose that Sti1/Hop might be easily accessible and bind efficiently to the mitochondrion-targeted CIRV replication proteins in cells, while the cellular Sti1/Hop cochaperone has a lesser chance to bind to the peroxisome-targeted TBSV and CNV replication proteins. Accordingly, live-cell imaging showed the relocalization of Sti1p to the mitochondria in the presence of CIRV p36, while Sti1p showed mostly cytosolic localization in yeast cells expressing the CNV p33 replication protein (Fig. 5). Thus, the difference in accessibility of Sti1/Hop could be the major mechanism restricting CIRV but not TBSV or CNV replication.

Mechanisms of Sti1p cochaperone-driven restriction of CIRV replication. Recruitment of the tombusvirus (+)RNA into replication requires selective binding by the tombusvirus replication proteins via recognition of a RNA recruitment element (named p33RE) within the polymerase gene sequence (54, 120). The same p33RE element is also required for the VRC assembly
and activation of the polymerase function of the replication protein (102, 121). The specific recognition of p33RE is performed by arginines within the RPR motif in p33/p92pol (122, 123). Therefore, binding of cellular factors to the RPR motif-containing region could block the ability of tombusvirus replication proteins to bind the viral repRNA, thus inhibiting the essential viral processes of repRNA recruitment, VRC assembly, and replicase activation (121, 124). Indeed, the TPR domain of Cpr7p binds to the RPR region in the tombusvirus p33/p92pol replication proteins and blocks the above viral processes, thus acting as a restriction factor (41). We find that Sti1p also binds to the RPR region in the CIRV p36/p95pol replication proteins (Fig. 4), and this could explain the strong in vitro inhibitory effect of recombinant Sti1p on CIRV replication based on mitochondrial preparations (Fig. 7). The CIRV p36 interaction with Sti1p also leads to the recruitment of Sti1p to punctate structures (mitochondrial membranes) in yeast cells, suggesting robust p36-Sti1p interaction in cells. Thus, direct interaction between the Sti1p and CIRV p36 might block viral RNA recruitment (Fig. 11, Model 1).

However, the picture on the mechanism of CIRV inhibition is likely more complex. This is because both the TPR1 and TPR2 regions of Sti1p bind to the RPR domain of CIRV p36/p95pol, yet the expression of TPR1 is inhibitory, while TPR2 is less effective in reducing CIRV accumulation in yeast or CIRV replication in vitro with mitochondrial preparations (Fig. 6 and 7). Thus, the binding to the RPR domain in p36/p95pol is unlikely enough for Sti1p to effectively inhibit p36 or p95pol functions.

Interestingly, Sti1p does not bind to the RPR region of the TBSV p33 replication protein. The binding between p33 and Sti1p involves the C-terminal region of p33 containing the p33-p33/p92 interaction sequence (Fig. 4G to H) and likely the corresponding sequence in p92pol. It is possible that this interaction is not robust or stable enough to interfere with p33/p92pol functions in cells. It is likely that p33 could readily interact with additional p33 molecules, while binding to Sti1p molecules by p33 might be less fa-

FIG 8 Comparison of the inhibitory effects of TPR-containing cellular proteins on CIRV replication in isolated mitochondria in vitro. (A) Denaturing PAGE analysis of the 32P-labeled repRNA products obtained in the replication assays with the isolated yeast mitochondrial preparations. The purified GST-tagged Sti1p, the yeast Cpr7p Cyp40-like cyclophilin, or the TPR domain of Cpr7p or Cns1p co-chaperone (8, 16, and 32 μM) was added in combination with purified recombinant CIRV MBP-p36 and MBP-p95 proteins and the TBSV-derived (+)repRNA to the isolated mitochondrial preparations to perform the in vitro replication assay. The synthesized full-length repRNA is marked by an arrow. See further details in the legend to Fig. 7. (B) Coomassie blue-stained SDS-PAGE was used for analysis of affinity-purified GST-tagged Sti1p, Cpr7p, and Cns1p. (C) The level of in vitro TBSV repRNA replication in total yeast cell extracts in the presence of purified TBSV p33 and p92 replication proteins and purified GST-tagged Sti1p, Cpr7p, the TPR domain of Cpr7p or Cns1p (16 and 32 μM). Each experiment was repeated three times.

FIG 9 Inhibition of CIRV replication by expression of the orthologous AtHop-1 in yeast. (A) A Split-ubiquitin MYTH assay was used to test intracellular interaction between CIRV p36 and the AtHop-1 ortholog of the yeast Sti1p. The bait p36 was coexpressed with the prey AtHop-1 protein in yeast. The SSA1 gene and the empty prey vector (NubG) were used as positive and negative controls, respectively. (B) Northern blot analysis of CIRV RNA accumulation in wt yeast overproducing the FLAG-tagged AtHop-1 or its TPR1-deletion derivative. Second panel, Northern blot analysis to demonstrate the comparable level of rRNA loading in the yeast samples. Third and fourth panels, Western blot analysis of CIRV His6-p36 and CIRV His6-p95 accumulation by anti-His antibody. Bottom panels, detection of the overproduced FLAG-AtHop-1 in yeast by Western blotting using anti-FLAG antibody and the Coomassie blue-stained SDS-PAGE as a loading control. Each experiment was repeated three times.
vored in cells. Indeed, Sti1p is not efficiently relocalized to punctate structures containing the p33 molecules in yeast cells (Fig. 5B). A similar situation was observed with Cpr6p Cyp40-like cyclophilin, which also binds to p33 within the C-terminal domain and does not inhibit p33 functions (41). Yet this rule is not general, since Cns1p cochaperone binds to the p33-p33/p92 interaction sequence in TBSV p33 replication protein and effectively inhibits TBSV replication in yeast (42) and in vitro (Fig. 8C). It seems that the intracellular distribution/accessibility of these TPR-containing host proteins might be a major factor in their ability to inhibit replication of different tombusviruses.

Although direct interaction between the RPR region of CIRV p36 and the TPR1 sequence in Sti1p might explain the inhibitory effect on CIRV replication (Fig. 11, Model 1), it is also possible that Sti1p limits the functions of subverted cellular factors, such as Hsp70, for its antiviral activity. Cytosolic Hsp70s are coopted by tombusviruses, and they are permanent residents in the tombusviral VRCs (39). This model is supported by the observation that, in spite of the binding of both TPR1 and TPR2 sequences to the RPR region of CIRV p36 (Fig. 3), only the expression of the Hsp70-interacting TPR1 region (89, 91) was able to robustly inhibit CIRV replication in yeast and in vitro (Fig. 6 and 7).
over, mutation within the TPR1 sequence (i.e., mutant C49Y) that debilitates the interaction with Hsp70, but not with p36 (Fig. 3) had lesser inhibitory effects on CIRV replication when expressed in yeast (Fig. 6). In contrast, a mutation (i.e., G325D) that affects interaction with Hsp90 did not interfere with the inhibitory function of Sti1p in vivo or in vitro. Based on these findings, we propose that the recruited Sti1p cochaperone inhibits the proviral function of the coopted cellular Hsp70 molecules during CIRV replication. For example, the predicted Sti1p-Hsp70 interaction during the formation of VRC or within the assembled VRC might inhibit the Hsp70-driven activation of the polymerase function of p95pol or other steps/functions (Fig. 11, Model 2).

The major role of the Sti1p cochaperone in eukaryotic cells is to bring the Hsp70-client protein complex together with the Hsp90 chaperone to facilitate robust refolding/activation of client proteins by the powerful Hsp90 system (91, 125, 126). This is facilitated by the ability of Sti1p to bind simultaneously to Hsp70 (via the TPR1 sequence) and Hsp90 (via the TPR2A region). However, based on our data, it is unlikely that this function of Sti1p is critical to inhibit CIRV replication. This is because deletion of the entire TPR2 domain from Sti1p did not eliminate the inhibitory function of Sti1p in vitro or in yeast (Fig. 6 and 7). Also, blocking the function of Hsp90 by applying a geldanamycin inhibitor in yeast had no effect on tombusvirus replication (data not shown), arguing against the functional role of Hsp90 in tombusvirus replication. Therefore, the direct effect of Sti1p on CIRV p36/p95pol and the coopted Hsp70 is the best suited to explain the current experimental data (Fig. 11).

Sti1/Hop is the first cellular restriction factor specifically affecting one tombusvirus (i.e., CIRV, which replicates in the mitochondria) but not other tombusviruses (TBSV and CNV, which both replicate in peroxisomal membranes). The previously identified TPR domain-containing cellular proteins, namely, Cpr7p and Cns1p, could inhibit the replication of all these tombusviruses (this work and references 41 and 42). Interestingly, all three cellular factors are part of the Hsp70/Hsp90 chaperone system, suggesting that they, at least in part, inhibit tombusvirus replication via regulating chaperone functions. Because the Hsp70/Hsp90 chaperone system is known to affect many viruses (reviewed in references 10 and 127), it is possible that the identified restriction factor activities of these TPR-containing cellular proteins might be functional against other viruses and pathogens.

Another use of Hop/Sti1 in host innate defense against pathogens is its role in the maturation and transport of rice chitin receptor OsCERK1, which is a pattern recognition receptor (PRR), against rice blast fungus (128). This function of Hop/Sti1 might link the functions of PRRs, small Rho-type GTPases, and resistance against pathogens. Sti1p is also known to affect prion propagation in yeast (129), and its expression is increased in simian virus 40 (SV40)-transformed MRC-5 fibroblasts and some tumor tissues (79, 130). Thus, Hop/Sti1 is emerging as a possibly key component in propagations of several infectious agents and innate defense responses of host cells.

**Summary.** The current and recent works (41, 42) with tombusviruses indicate that some members of the large family of TPR-containing proteins might act as cell-intrinsic restriction factors of tombusviruses. The list includes the Hop-like Sti1p and Ttc4 oncogene-like Cns1p cochaperones and Cyp40-like Cpr7p cyclophilin. Yet, based on the yeast Cyp40-like Cpr6p cyclophilin (41), we already know that not all TPR-containing proteins are viral restriction factors in spite of their abilities to interact with tombusvirus replication proteins. Since many TPR-containing proteins are expressed in all eukaryotes, it will be important to identify all the members of this cellular protein family that act as restriction factors during the replication of tombusvirus and other (+)RNA viruses.
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