5-2015

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Digital Object Identifier (DOI)
http://dx.doi.org/10.1128/JVI.03711-14

Notes/Citation Information
Published in *Journal of Virology*, v. 89, no. 10, p. 5714-5723.

JVI Journals.ASM.org

Activation of *Tomato Bushy Stunt Virus* RNA-Dependent RNA Polymerase by Cellular Heat Shock Protein 70 Is Enhanced by Phospholipids *In Vitro*

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ABSTRACT

Similar to other positive-strand RNA viruses, tombusviruses are replicated by the membrane-bound viral replicase complex (VRC). The VRC consists of the p92 virus-coded RNA-dependent RNA polymerase (RdRp), the viral p33 RNA chaperone, and several co-opted host proteins. In order to become a functional RdRp after its translation, the p92 replication protein should be incorporated into the VRC, followed by its activation. We have previously shown in a cell-free yeast extract-based assay that the activation of the *Tomato bushy stunt virus* (TBSV) RdRp requires a soluble host factor(s). In this article, we identify the cellular heat shock protein 70 (Hsp70) as the co-opted host factor required for the activation of an N-terminally truncated recombinant TBSV RdRp. In addition, small-molecule-based blocking of Hsp70 function inhibits RNA synthesis by the tombusvirus RdRp *in vitro*. Furthermore, we show that neutral phospholipids, namely, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), enhance RdRp activation *in vitro*. In contrast, phosphatidylglycerol (PG) shows a strong and dominant inhibitory effect on *in vitro* RdRp activation. We also demonstrate that PE and PC stimulate RdRp-viral plus-strand RNA [(+)RNA] interaction, while PG inhibits the binding of the viral RNA to the RdRp. Based on the stimulatory versus inhibitory roles of various phospholipids in tombusvirus RdRp activation, we propose that the lipid composition of targeted subcellular membranes might be utilized by tombusviruses to regulate new VRC assembly during the course of infection.

IMPORTANCE

The virus-coded RNA-dependent RNA polymerase (RdRp), which is responsible for synthesizing the viral RNA progeny in infected cells of several positive-strand RNA viruses, is initially inactive. This strategy is likely to avoid viral RNA synthesis in the cytosol that would rapidly lead to induction of RNA-triggered cellular antiviral responses. During the assembly of the membrane-bound replicase complex, the viral RdRp becomes activated through an incompletely understood process that makes the RdRp capable of RNA synthesis. By using TBSV RdRp, we show that the co-opted cellular Hsp70 chaperone and neutral phospholipids facilitate RdRp activation *in vitro*. In contrast, phosphatidylglycerol (PG) has a dominant inhibitory effect on *in vitro* RdRp activation and RdRp-viral RNA interaction, suggesting that the membranous microdomain surrounding the RdRp greatly affects its ability for RNA synthesis. Thus, the activation of the viral RdRp likely depends on multiple host components in infected cells.

Replication of plus-strand RNA [(+)RNA] viruses requires the assembly of the viral replicase on subcellular membrane surfaces. The viral replicase complex (VRC) consists of virus-coded RNA-dependent RNA polymerase (RdRp), viral auxiliary replication proteins, and a number of co-opted host proteins (1–7). Interestingly, the viral RdRp is assumed to be inactive in the cytosol to prevent the formation of a viral double-stranded RNA (dsRNA) intermediate that could trigger efficient gene silencing and RNA-induced innate immunity (8–10). Therefore, the VRC assembly, including the activation of the viral RdRp, could be a critical regulatory step during the infection cycle.

The tombusvirus VRCs are housed in membranous spherule structures, which are membrane invaginations into peroxisomes or mitochondria in the cases of *Tomato bushy stunt virus* (TBSV) and *Carnation Italian ringspot virus* (CIRV), respectively (11–15). The tombusvirus VRCs contain the virus-coded p92 RdRp and p33 RNA chaperone and the dsRNA intermediate, as well as co-opted cellular proteins, such as the heat shock protein 70 (Hsp70) molecular chaperone (coded for by yeast [*Saccharomyces cerevisiae*] *SSA1/2*), glyceraldehyde-3-phosphate dehydrogenase (GAPDH [coded for by *TDH2/3* in yeast]), Cdc34p E2 ubiquitin-conjugating enzyme, eukaryotic translation elongation factor 1A (eEF1A), eEF1Bγ, the Vps4p ESCRT (endosomal sorting complex required for transport) protein, and DDX3-like Ded1p and eIF4AIII-like RH2 DEAD box helicases (16–30). Hsp70, eEF1A, Cdc34p, and ESCRT proteins are involved in the assembly of the tombusviral VRC, while other subverted RNA-binding proteins (eEF1A, eEF1Bγ, GAPDH, Ded1p, and RH2) facilitate viral RNA synthesis (1, 3, 18, 31). Tombusvirus replication also depends on lipids, such as sterols and phospholipids, and oxysterol-binding

Received 26 December 2014 **Accepted** 8 March 2015

Accepted manuscript posted online 11 March 2015

Citation Pogany J, Nagy PD. 2015. Activation of *Tomato bushy stunt virus* RNA-dependent RNA polymerase by cellular heat shock protein 70 is enhanced by phospholipids *in vitro*. J Virol 89:5714–5723. doi:10.1128/JVI.03711-14.

Editor: A. Simon

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doi:10.1128/JVI.03711-14

proteins (ORPs) that help the formation of the sterol- and phospholipid-rich microenvironment needed for VRC formation (32–34).

Similar to TBSV, several (+)RNA viruses also have RdRps that need activation before RNA synthesis initiates. The list includes *Cucumber necrosis virus* (CNV) p92, *Brome mosaic virus* (BMV) $2a^{pol}$, P2 of *Alfalfa mosaic virus*, *Tomato mosaic virus* (ToMV) 180K, the *Hepatitis C virus* (HCV) NS5B RdRp proteins, and nodavirus protein A (35–39). After activation, many viral RdRps could be purified and their activities characterized in template-dependent *in vitro* assays (40).

To dissect the roles of viral and host factors during the TBSV RdRp activation step, we have previously developed a simplified system based on N-terminally truncated TBSV $p92^{pol}$ RdRp that requires fewer components than the complete $p92^{pol}$/p33 replicase for activation of the RNA synthesis function (41). Unlike the full-length TBSV $p92^{pol}$, the N-terminally truncated TBSV p92-Δ167N RdRp does not require the p33 replication cofactor or cellular membranes to produce RNA products on the added viral (+)RNA templates *in vitro* (18, 41). The activation of TBSV p92-Δ167N RdRp still needs a soluble host factor(s) and a *cis*-acting viral (+)RNA element. Accordingly, the *Escherichia coli*-expressed and purified TBSV p92-Δ167N RdRp shows little RdRp activity in the absence of the soluble fraction of yeast cell-free extract (CFE), indicating that a subverted host factor(s) is critical for RdRp activation (41).

In this work, we show that the purified cellular Ssa1p Hsp70 chaperone is able to activate the RdRp function of p92-Δ167N *in vitro*. In addition, phospholipids, such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC), could further enhance the RdRp activity of TBSV p92-Δ167N *in vitro*. PE and PC also promote the RNA binding capability of TBSV p92-Δ167N with the viral (+)RNA template. Interestingly, other phospholipids, such as phosphatidylglycerol (PG), repress the activation of TBSV p92-Δ167N and strongly inhibit RdRp-RNA interactions. Altogether, the presented data indicate that the co-opted cellular Hsp70 and phospholipids are important factors regulating viral functions, including the RdRp activation and replication protein-viral RNA interactions, which are critical for VRC assembly in infected cells.

MATERIALS AND METHODS

Affinity purification of recombinant tombusvirus replicase protein from yeast for mass spectrometry analysis. FLAG-tagged p92-Δ167N was expressed from the copper-inducible *CUP1* promoter at 29°C. Following protein induction, yeast proteins were cross-linked using formaldehyde according to reference 42. Yeast cells were broken in breaking buffer (30 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA) containing β-mercaptoethanol and yeast protease inhibitor cocktail, using a Fast Prep machine. Low-speed centrifugation was used at 500 × *g* for 5 min to remove cellular debris. After centrifugation, the NaCl concentration of the supernatant was adjusted to 0.5 M, Triton X-100 was added to a 1% final concentration, and the mixture was centrifuged at 43,000 × *g* for 30 min. The supernatant was loaded on an equilibrated FLAG-affinity column. The column was washed 3 times with wash buffer (breaking buffer containing 0.5 M NaCl and 1% Triton). The bound proteins were eluted with the FLAG-tagged peptide in the wash buffer containing 0.5% Triton. To reverse cross-linking, the purified protein preparations were boiled for 30 min in SDS-PAGE loading dye. The protein sample was run into the separating gel of an SDS-PAGE gel with a very short run. A narrow band containing the proteins was excised from the gel and submitted for mass spectrometry analysis.

Preparation of phospholipids. The phospholipids used in these experiments included 18:1 PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), 18:1 PC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), 18:1 PS (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine), 18:1 PG [1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol)], 18:1 CA (1′,3′-bis [1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol), 18:1 lysoPE (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine), 18:1 lysoPC (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine), L-α-phosphatidylinositol-3-phosphate (PI3P), and cholesterol and were obtained from Avanti Polar lipids and stored in chloroform.

Lipids were added into glass vials and subsequently dried under vacuum for 2 to 3 h. Dimethyl sulfoxide (DMSO) was added to each vial to make up the lipid concentration to 700 μM, followed by extensive vortexing. Two microliters of each lipid was used in a 20-μl *in vitro* assay, unless noted otherwise in the figure legend. The final DMSO concentration was kept at 10% in all *in vitro* reactions except when noted otherwise.

Expression and purification of recombinant tombusvirus replicase proteins from *E. coli*. The maltose-binding protein (MBP)-tagged TBSV p92-Δ167N replicase protein was expressed in *E. coli* and purified according to reference 41. MBP-p33C is an N-terminally truncated TBSV p33 protein expressed as an MBP fusion protein (43). p92-Δ167N RdRp is an N-terminally truncated TBSV-p92 protein (41).

Preparation of RNA templates for *in vitro* RdRp and replicase assays. We produced the following set of RNA templates: DI-72 minus-strand RNA [(−)RNA] (44) and DI-mini [(+)RNA] (35). The T7-based *in vitro* transcription reaction was done after the PCR amplification and purification of PCR products (45).

EMSA. Electrophoretic mobility shift assay (EMSA) experiments were done according to reference 43. The ^{32}P-labeled RII(+)SL hairpin sequence was used for the EMSAs with MBP-p33C (43). DI-mini (+)RNA was used for the gel shift experiments with p92-Δ167N RdRp. For the latter EMSAs, the soluble fraction of yeast CFE was also added as described previously (41).

***In vitro* RdRp assay.** Recombinant affinity-purified MBP-p92-Δ167N RdRp was incubated with the yeast soluble fraction (2 μl) in a buffer containing 50 mM HEPES-KOH (pH 7.4), 50 mM potassium acetate, 5 mM magnesium acetate, 0.2 M sorbitol, 0.4 μl actinomycin D (5 mg/ml), 2 μl of 150 mM creatine phosphate, 2 μl of 10 mM ATP, CTP, and GTP and 0.25 mM UTP, 0.1 μl of [^{32}P]UTP, 0.2 μl of 10 mg/ml creatine kinase, 0.2 μl of RNase inhibitor, 0.2 μl of 1 M dithiothreitol, and 0.5 μg RNA transcript in a 20-μl reaction mixture.

The reaction mixture was incubated at 25°C for 3 h, followed by termination by the addition of 110 μl stop buffer (1% sodium dodecyl sulfate [SDS], 0.05 M EDTA [pH 8.0]). Then phenol-chloroform extraction was performed, followed by isopropanol-ammonium acetate precipitation, and a washing step with 70% ethanol as described earlier (41).

***In vitro* RdRp activation assay.** Yeast cell-free extract (CFE) was prepared according to reference 18. The CFE was centrifuged at 4°C for 20 min at 42,000 × *g* to obtain the soluble (supernatant) CFE fraction. FLAG-tagged yeast Ssa1p (Hsp70) was overexpressed in yeast and purified using an anti-FLAG column according to reference 18. The HSP70 inhibitor (MKT-077) was dissolved in DMSO (46) and added to the *in vitro* reaction mixture in 50 and 100 μM final concentrations. DMSO was added to the control reaction mixtures. The RdRp activation assay was conducted as the standard *in vitro* RdRp assay, except that the soluble fraction was replaced with the FLAG-tagged purified Ssa1p Hsp70 and 2 μl phospholipids dissolved in DMSO. As a control, we added 2 μl DMSO to the reaction mixture lacking phospholipids.

Replication assay based on affinity-purified replicase. FLAG-tagged CNV replicase complex was purified from yeast expressing FLAG-CNV92, FLAG-CNV33, and DI-72 replicon RNA as described previously (28, 35, 36).

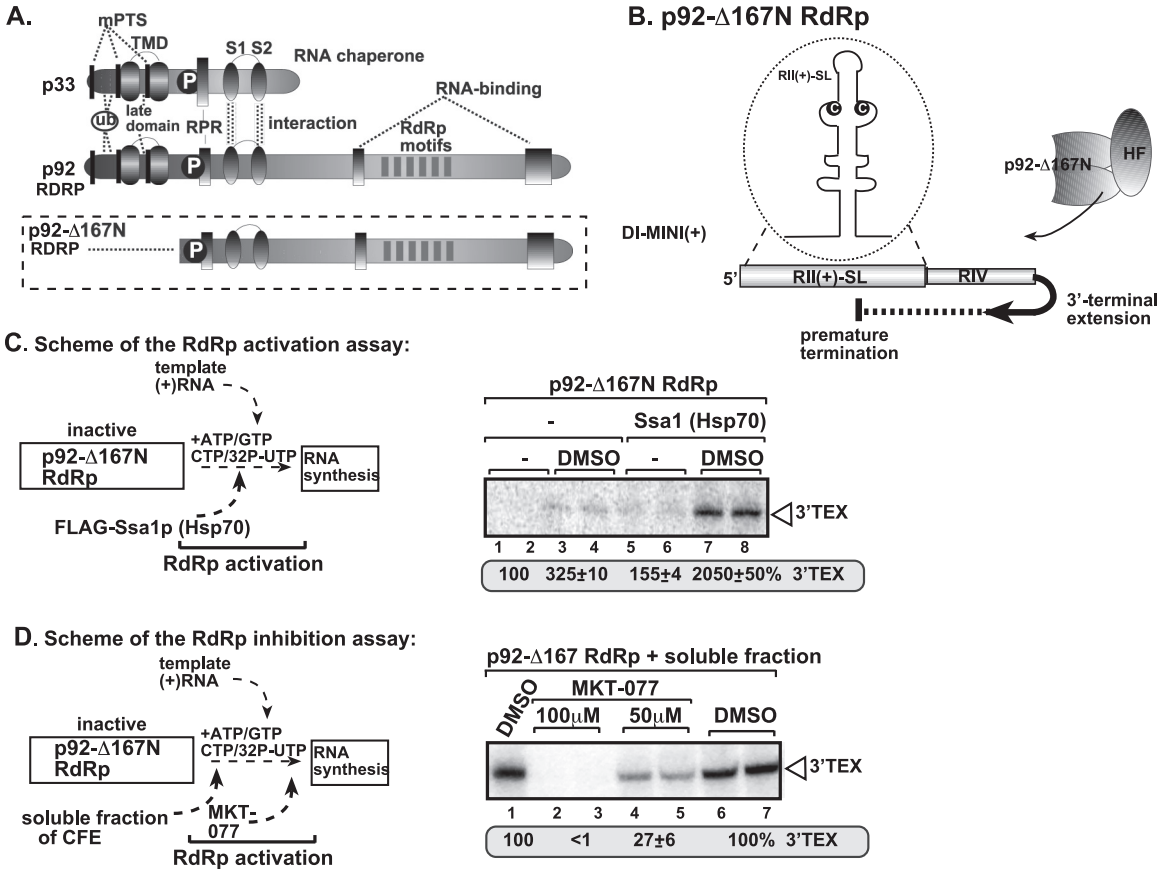

FIG 1 *In vitro* activation of the RdRp function of the N-terminally truncated p92^{pol} replication protein depends on cellular Hsp70 chaperone. (A) Schematic representation of the known domains in the p92-Δ167N RdRp. The missing 167-amino-acid (aa) N-terminal sequence is indicated by a dotted line. The full-length TBSV p33 and p92 replication proteins are shown at the top. The N-terminal segment in the TBSV p92^{pol} contains the same sequence as p33 due to the overlapping expression strategy of TBSV genome, while the C-terminal region of p92^{pol} carries the RdRp domain and two RNA-binding sequences. The various domains in the shared sequences are termed as follows: mPTS, peroxisomal membrane targeting sequences; ub, monoubiquitinylation sites; TMD, transmembrane domains; late domain, sequence recognized by the ESCRT factors; P, phosphorylation sites; RPR, arginine-proline-rich RNA-binding domain, required for selective recognition of RII(+)-SL sequence in the viral (+)RNA. S1 and S2 are subdomains of the p33-p33/p92 interaction domain. (B) Based on previous work, the activated recombinant p92-Δ167N RdRp protein produces a 3′-terminal extension (3′-TEX) product and terminates prematurely at the hairpin structure, as shown. Schematic representation of DI-mini (+)RNA template with the known *cis*-acting p33RE RNA element [RII(+)-SL carrying the critical C·C mismatch required for template recognition by the TBSV replication proteins] and the short RIV(+), which harbors *cis*-elements for minus-strand synthesis (but missing the ss4 region). (C) Scheme of the *in vitro* RNA synthesis assay with DI-mini (+)RNA and p92-Δ167N RdRp protein. (Right panel) Denaturing PAGE analysis of the ^{32}P-labeled RNA products obtained in an *in vitro* assay with recombinant p92-Δ167N RdRp. The samples contained or lacked affinity-purified Ssa1p Hsp70 protein (13 pmol) with or without DMSO. The 3′-TEX product is pointed at with an open arrowhead. The amounts of 3′TEX products were estimated using the Imagequant software. (D) Scheme of the *in vitro* RNA synthesis assay in the presence of an allosteric Hsp70 inhibitor (MKT-077). The assay also contained DI-mini (+)RNA and p92-Δ167N RdRp protein in the presence of only the soluble fraction of CFE (used in the same amount in each sample). See further details in the legend for panel C. DMSO, which was used as a solvent for MKT-077, was used as a control. Each experiment was repeated two or three times.

RESULTS

Mass spectrometry-based identification of yeast proteins copurified with a truncated p92^{pol} replication protein. The recombinant TBSV p92-Δ167N (Fig. 1A), which is inactive in the RdRp buffer, is activated *in vitro* by the addition of the soluble fraction of yeast CFE, suggesting that this fraction contains a cellular protein(s) required for RdRp activation (41). To identify the putative soluble factor(s) involved in RdRp activation in the yeast CFE, we expressed FLAG-tagged p92-Δ167N RdRp in yeast, followed by FLAG affinity purification of the RdRp protein. We then performed mass spectrometry analysis of the copurified yeast proteins in the affinity-purified p92-Δ167N preparation, which revealed the presence of at least 13 yeast proteins (Table 1). Altogether, we have identified five yeast proteins that have previously been shown to be part of the tombusvirus VRC, including Ssa1p and Ssa2p Hsp70 chaperones, Tdh3 (GAPDH), Tef1p (eEF1A), and Pdc1p (19, 25, 28). An additional two yeast proteins have been identified in previous high-throughput screens, namely, Cdc19p and Pgk1p (47, 48). The remaining 6 yeast proteins have not been identified in previous screens (Table 1). Since the copurified yeast proteins included five heat shock protein molecular chaperones, it is possible that one or more of these cellular chaperones could participate in the activation of the tombusvirus RdRp during infections. We have decided to further study the role of the cytosolic Hsp70 (the SSA1-4 subfamily in yeast) based on its functions in tombusvirus VRC assembly and localization of p33 and p92^{pol} replication proteins in subcellular membranes (16–18). Ssa1/2 are permanent residents of the tombusvirus VRCs,

TABLE 1 Yeast proteins copurified with TBSV p92-Δ167N

Protein	No. of peptides	Function
Ssa1[a]	18	Member of HSP70 family, cytosolic ATPase involved in protein folding
Ssa2[a]	17	Member of HSP70 family, 98% identical to paralog Ssa1p
Eno2	4	Enolase II, phosphopyruvate hydratase
Tdh3[a]	5	GAPDH
Ssb1	4	Member of HSP70 family, cytoplasmic ATPase, ribosome-associated chaperone
Hsc82	7	Cytoplasmic chaperone of Hsp90 family
Tef1[a]	3	Translational elongation factor EF-1 alpha
Cdc19[b]	5	Pyruvate kinase
Eft1	2	Elongation factor 2 (EF-2), catalyzes ribosomal translocation during protein synthesis
Pgk1[b]	1	3-Phosphoglycerate kinase, ATP synthesis
Hsp104	1	Disaggregase, heat shock protein
Leu1	1	Isopropylmalate isomerase, catalyzes second step in leucine biosynthesis pathway
Pdc1[a]	1	Major of 3 pyruvate decarboxylase isozymes

[a] Present in the tombusvirus VRC.
[b] Identified as a host factor for TBSV previously.

but they do not seem to affect minus- or plus-strand synthesis (16, 19, 30).

Ssa1p Hsp70 activates the RdRp function of the TBSV p92^{pol} replication protein *in vitro*. We first tested a purified yeast Ssa1p Hsp70 preparation in combination with p92-Δ167N and a template RNA (Fig. 1B) in an *in vitro* RNA synthesis assay (Fig. 1C). This assay demonstrated that the purified Ssa1p was able to activate the RdRp function of TBSV p92-Δ167N in the presence of DMSO (Fig. 1C, lanes 7 to 8). As expected, TBSV p92-Δ167N did not show RdRp activity in the absence of Ssa1p and DMSO in the RdRp buffer (Fig. 1C, lanes 1 to 2). As we have demonstrated before (41) and schematically shown in Fig. 1B, the TBSV p92-Δ167N synthesized ^{32}P-labeled 3′-terminal extension (3′-TEX) product using the DI-mini template (while no *de novo*-initiated products were detected [data not shown]).

To confirm that indeed Hsp70 is the soluble cellular factor in the yeast CFE required for activation of p92-Δ167N, we added a specific Hsp70 inhibitor, called MKT-077 (46), to the RdRp activation assay containing p92-Δ167N, the soluble fraction of yeast CFE, and DI-mini template (Fig. 1D). The Hsp70 inhibitor strongly inhibited the activity of p92-Δ167N *in vitro* (Fig. 1D, compare lanes 2 to 5 with 6 to 7). Altogether, these data have established an essential role for the cellular Hsp70 in the *in vitro* activation of the TBSV RdRp function.

Neutral PE and PC phospholipids enhance the activity of the TBSV RdRp *in vitro*. Although Ssa1p was able to activate the RdRp function of TBSV p92-Δ167N, the observed level of RNA synthesis is only ~20 to 30% of that measured with the whole CFE (data not shown), suggesting that other factors will also contribute to enhancement of TBSV RdRp activity. To this end, we have tested phospholipids because TBSV replicates in a membranous environment (spherule-like structures in the peroxisomal membrane), and depletion of phospholipids in cellular membranes has adverse effects on TBSV replication (14, 26, 33, 49).

We have decided to study the effects of the most abundant phospholipids in cells, such as the neutral phosphatidylethanolamine (PE) and phosphatidylcholine (PC), phosphatidylinositol-3-phosphate (PI3P), and the negatively charged phosphatidylglycerol (PG) and cardiolipin (CA or CL). While these phospholipids alone did not activate the RdRp function of p92-Δ167N (not shown), we observed ~2.5-fold stimulation of RNA synthesis by p92-Δ167N in the presence of PE and PC in combination with Ssa1p Hsp70 and DMSO (Fig. 2A, lanes 3 to 6 versus 11 and 12). On the other hand, PI3P did not enhance the activity of p92-Δ167N, while PG and the total lipids from *E. coli* inhibited the RdRp activity (Fig. 2A). In contrast, these phospholipids affected the *in vitro* activity of the purified tombusvirus replicase, which has been assembled and activated in yeast prior to isolation, by only 15 to 30% (Fig. 2B). This effect on RdRp activity is negligible in comparison to the effects of these phospholipids on RdRp activation. Altogether, our results demonstrate that neutral phospholipids are not essential but stimulate the activation of the RdRp *in vitro*.

The acidic PG phospholipid is a potent inhibitor of the TBSV RdRp activity *in vitro*. The above-described experiments have indicated that the addition of acidic PG inhibited the RdRp activation (Fig. 2A). To characterize the inhibitory role of PG on the RdRp activation, we used increasing concentrations of PG in the RdRp activation assay. Interestingly, 14 μM PG completely blocked the RdRp activity, while 3 μM PG reduced the activity to ~40% (Fig. 3A, lanes 4 to 7). Thus, PG seems to act as a potent inhibitor of RdRp activation.

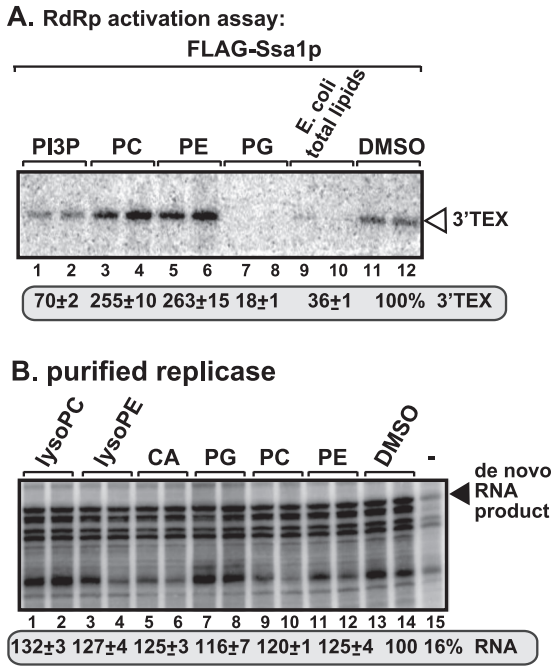

FIG 2 Effect of phospholipids on the *in vitro* RdRp activity of the p92-Δ167N RdRp protein. (A) PAGE analysis of the ^{32}P-labeled RNA products obtained in an *in vitro* assay with purified p92-Δ167N RdRp protein in the presence of purified Ssa1 Hsp70 (13 pmol) and DI-mini (+)RNA template. In addition, samples also contained various phospholipids as shown (70 μM). See further details in Fig. 1. Each experiment was repeated two times. (B) Denaturing PAGE analysis of the ^{32}P-labeled RNA products obtained in an *in vitro* assay with FLAG-affinity-purified CNV replicase in the presence of the buffer, DMSO, or the shown phospholipids (70 μM). The template used was DI-72 (−)RNA, which leads to the production of *de novo*-initiated full-length (top band, marked with an arrowhead and quantified) and internally initiated shorter products. Note that samples contained 0.1% detergent required for the purification of the replicase preparation.

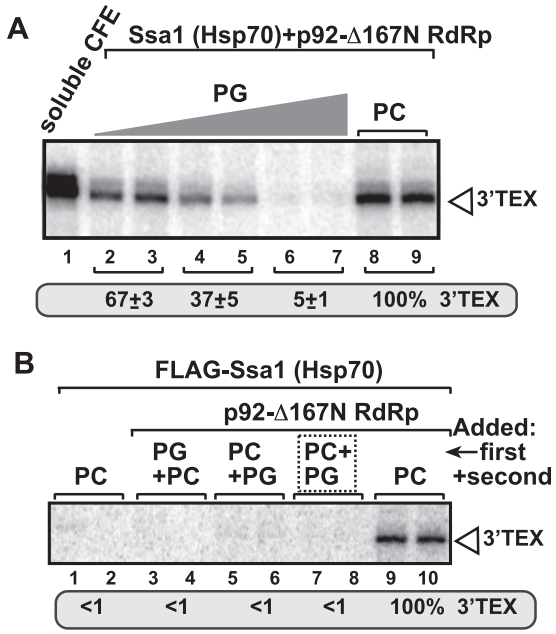

FIG 3 Dominant inhibitory effect of PG on the *in vitro* RdRp activity of the p92-Δ167N RdRp protein. (A) PAGE analysis of the ^{32}P-labeled RNA products obtained in an *in vitro* assay with purified p92-Δ167N RdRp protein in the presence of purified Ssa1 Hsp70 (13 pmol) and DI-mini (+)RNA template. In addition, samples also contained PG (0.6, 3, or 14 μM) or PC (14 μM) as shown. See further details in Fig. 1. Each experiment was repeated two times. (B) PAGE analysis of the ^{32}P-labeled RNA products obtained in an *in vitro* assay with purified p92-Δ167N RdRp protein. Note that PG and PC were used in a 1:1 ratio, but either PG or PC was added first to the assay (preincubation with PG and PC in lanes 3 and 4 and lanes 5 and 6, respectively), followed by addition of the other phospholipid as shown. The samples in a dotted box had PG and PC added at the same time (lanes 7 and 8). See further details in panel A.

To test if the inhibitory effect of PG is dominant over the proviral activity of PC, we mixed PG with PC in 1:1 ratio in the RdRp activation assay. This resulted in complete inhibition of the RdRp activity *in vitro* (Fig. 3B, lanes 7 and 8 versus 9 and 10). Preincubation of the p92-Δ167N RdRp preparation with PC, followed by addition of PG (in the same amount as PC) 5 min later, did not prevent the inhibitory effect of PG on RdRp activation (Fig. 3B, lanes 5 to 6). Altogether, these data indicate that PG's inhibitory effect on the p92-Δ167N RdRp activity is dominant *in vitro*.

PE and PC facilitate viral RNA binding by the RdRp. One of the interesting features of the TBSV p92-Δ167N RdRp is the selective binding to the *cis*-acting RII(+)-SL (Fig. 1B), which is required for the activation of the TBSV RdRp *in vitro* (41). In addition, RII(+)-SL serves as an RNA recognition element by p33 (p33RE) required for the selection and recruitment of the viral (+)RNA for replication in cells and in cell extract (18, 50).

To test if phospholipids affect the binding of the TBSV p92-Δ167N RdRp to the viral (+)RNA carrying the critical p33RE sequence, we performed EMSA (18). Compared to the buffer control, PC and PE stimulated RNA binding by the TBSV RdRp by up to ~3-fold (Fig. 4A, lanes 4 to 7 versus 2 and 3), while PS did not have an effect (lanes 10 and 11). Similarly, lysoPE had stimulatory effect, while lysoPC and cholesterol did not (Fig. 4A). On the contrary, PG completely blocked viral RNA binding by the TBSV p92-Δ167N RdRp (Fig. 4A, lanes 8 and 9). Inhibitory effects were also observed for CA and PI4P phospholipids (Fig. 4A). Overall, these data suggest that various phospholipids have major effects, which can be either stimulatory or inhibitory, on the ability of the TBSV p92-Δ167N RdRp to bind to the viral (+)RNA template *in vitro*.

Since PG was inhibitory on the binding of the viral RNA to the RdRp, we analyzed if this effect is dominant over the stimulatory effect of PE. Using PG and PE in various ratios in EMSA, we observed remarkable inhibition by PG on the RNA binding by the p92-Δ167N RdRp when applied in a 1:1 ratio with the stimulatory PE (Fig. 4B, lanes 9 and 10 versus 1 and 2). Thus, PG acts dominantly over the stimulatory effect of PE in inhibition of template binding by the TBSV p92-Δ167N RdRp.

Since different tombusviruses replicate in different subcellular membranes, such as peroxisomal for TBSV and mitochondrial for CIRV (11–15), whose membranes differ in lipid composition, we tested the effect of various phospholipids on the abilities of TBSV p92-Δ167N RdRp versus the corresponding CIRV p95-Δ200N RdRp *in vitro*. The *in vitro* activation of CIRV p95-Δ200N RdRp depends on factors similar to those of TBSV p92-Δ167N RdRp (41). The EMSA revealed comparable profiles for the stimulatory effect of PE, PC, and lysoPE and a strong inhibitory effect of PG on viral RNA binding by these RdRp proteins (Fig. 5). Thus, the roles of given phospholipids in enhancing the activation of these tombusvirus RdRps are rather similar. Accordingly, these viruses alter the phospholipid content of particular subcellular membranes where their replication takes place by redistributing PE to the sites of replication (72).

Phospholipids also affect viral RNA binding by the viral p33 replication protein. Cellular membranes and phospholipids are required for the assembly of the tombusviral replicase complex in cells and *in vitro* (18, 33, 49). The virus-coded p33 replication protein is the major component of the tombusvirus VRC, and it is required for VRC assembly, viral (+)RNA recruitment, and stimulation of *de novo* viral RNA synthesis by the RdRp (18, 41, 51–53). To test if various phospholipids could affect the binding of p33 to the viral (+)RNA, we performed an EMSA. Interestingly, PE and PC stimulated the ability of p33 to bind to the viral (+)RNA (Fig. 6A and B). However, unlike in the case of the TBSV p92-Δ167N RdRp, PG and CA did not inhibit p33-RNA interaction (Fig. 6A) when p33 was added at high concentrations, while PG and CA inhibited this activity at a lower concentration of p33 (Fig. 6B). When p33 was present at the highest concentration, none of the phospholipids had effects on p33-RNA interaction (Fig. 6A, lanes 3 to 10). Altogether, phospholipids affect the ability of p33 to bind to the viral (+)RNA, with PE and PC having stimulatory effects while PG and CA manifest inhibitory effects.

DISCUSSION

Critical role of co-opted Hsp70 in activation of TBSV RdRp. Tombusvirus replication leads to the production of dsRNA intermediates that are used to make an excess amount of new (+)RNA progeny in infected cells (29). However, dsRNA is a strong inducer of RNA silencing, an innate antiviral response of the host cells (8–10). To avoid the rapid induction of the RNA silencing response, tombusviruses, similar to other (+)RNA viruses, are assumed to hide their dsRNA intermediates in spherule-like membrane structures, which likely make the dsRNA inaccessible to the RNA silencing machinery (26, 30). Therefore, it is critical for tombusviruses to produce an inactive RdRp protein during transla-

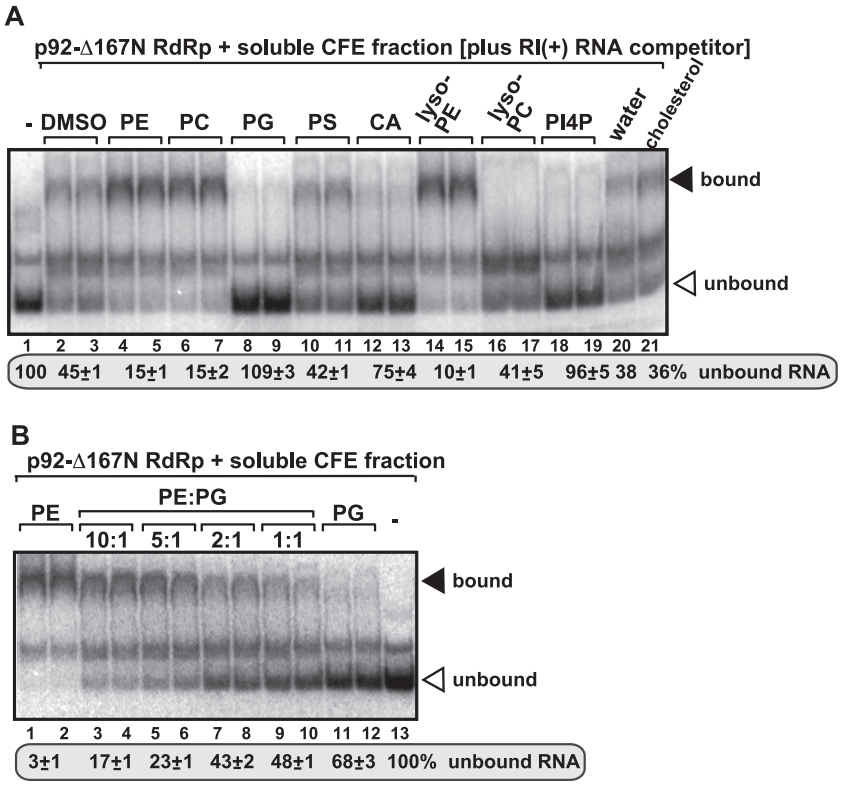

FIG 4 Effect of phospholipids on the *in vitro* RNA-binding activity of the p92-Δ167N RdRp protein. (A) EMSA analysis of the *in vitro* binding of purified TBSV p92-Δ167N RdRp to ^{32}P-labeled DI-mini (+)RNA [carrying the RII(+)-SL sequence] (~0.1 pmol). The assay contained the shown phospholipids added in comparable amounts (70 μM). The free or the RdRp-bound ssRNA was separated on nondenaturing 5% Tris-acetate-EDTA (TAE) acrylamide gels. Each experiment was repeated at least two times. (B) Effect of various ratios of PE to PG on the *in vitro* RNA-binding activity of the p92-Δ167N RdRp protein. (B) EMSA analysis of the *in vitro* binding of purified p92-Δ167N RdRp to ^{32}P-labeled RNA in the presence of mixed phospholipids. The assay contained various ratios of PE and PG in comparable amounts of total phospholipids (70 μM). See further details in panel A.

tion occurring in the cytosol, followed by RdRp activation only during VRC assembly in membranous structures. Accordingly, similar to the yeast- or plant-expressed p92^{pol} (when expressed in the absence of p33 and the viral RNA), the *in vitro*-translated p92^{pol} is inactive as an RdRp protein, albeit it can bind to the viral RNA (36, 54).

However, the viral RdRp must be activated to support robust and efficient viral RNA synthesis in the membranous structures. In this article, we show evidence that the activation of the TBSV p92-Δ167N requires the co-opted cellular Hsp70 protein, called Ssa1p in yeast. Ssa1p and the homologous Ssa2p are recruited from the cytosol to the tombusvirus replicase via binding to p33 and p92, and these Hsp70s are permanent members of the VRC (19, 30), making them well suited to perform the RdRp activation function. Also, Ssa1p and Ssa2p were identified in our affinity-purified p92-Δ167N RdRp preparation (Table 1), suggesting that

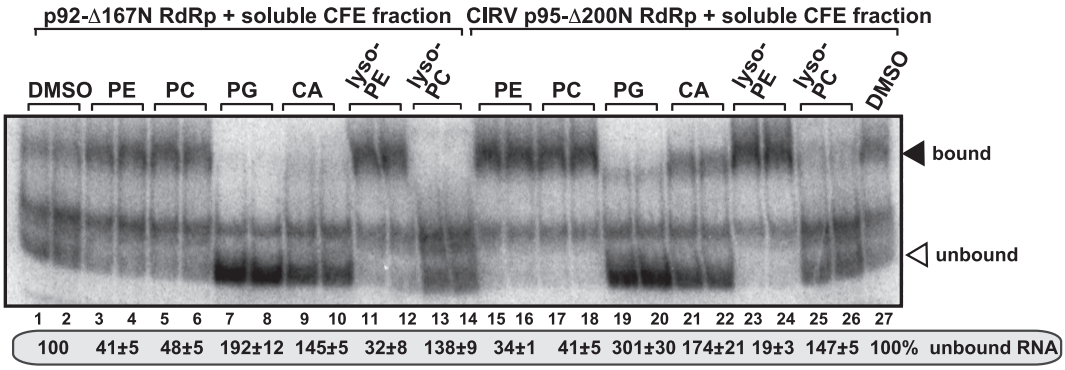

FIG 5 Comparable effects of phospholipids on the *in vitro* RNA-binding activities of the CIRV p95-Δ200N and the TBSV p92-Δ167N RdRp proteins. EMSA analysis of the *in vitro* binding of purified CIRV p95-Δ200N RdRp or TBSV p92-Δ167N RdRp to ^{32}P-labeled DI-mini (+)RNA template (~0.1 pmol). The assay contained the shown phospholipids added in comparable amounts (70 μM). The free or the RdRp-bound ssRNA was separated on nondenaturing 5% acrylamide gels. Each experiment was repeated at least two times.

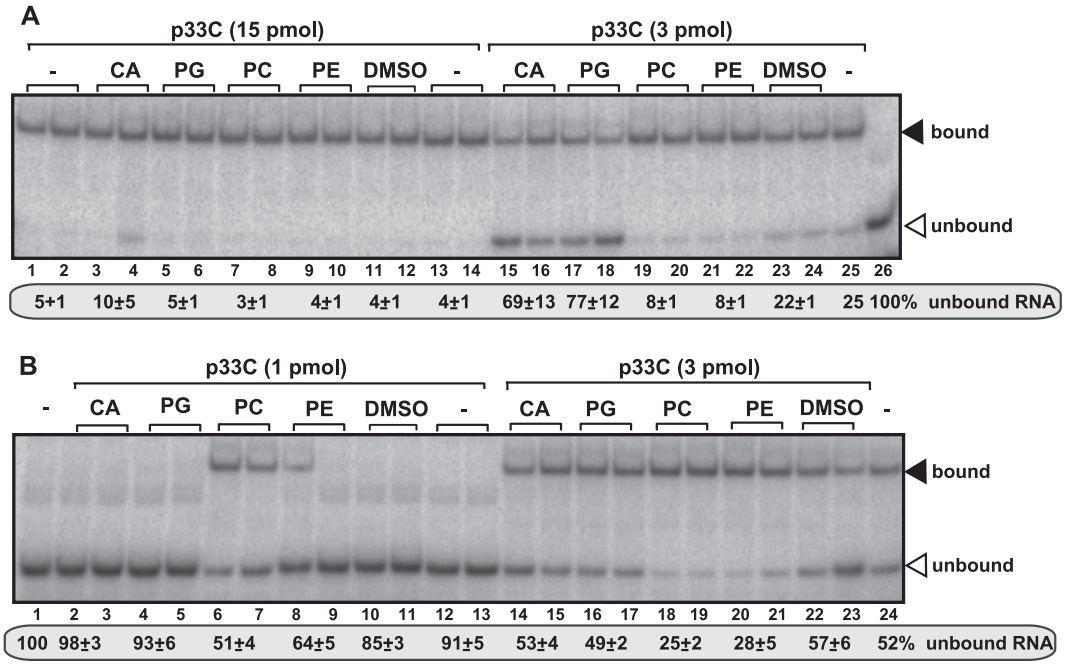

FIG 6 Effects of phospholipids on the *in vitro* RNA-binding activity of the TBSV p33C replication protein. (A) EMSA analysis of the *in vitro* binding of purified TBSV p33C (p33 carrying the soluble C-terminal half) to ^{32}P-labeled wild-type RII(+)-SL template (~0.1 pmol). The assay contained the shown phospholipids added in comparable amounts (70 μM). The free or the RdRp-bound ssRNA was separated on nondenaturing 5% acrylamide gels. Each experiment was repeated at least two times. (B) Similar EMSA analysis as in panel A, except the p33C was preincubated with the shown phospholipids (70 μM each) for 5 min prior to the addition of the ^{32}P-labeled RII(+)-SL RNA probe.

the viral RdRp and Hsp70 form a complex in yeast. The binding of Hsp70 likely leads to refolding of the originally inactive RdRp protein, rendering it competent for RNA synthesis. The newly discovered RdRp activation function of Hsp70 adds to the list of tombusviral functions already known to require Hsp70, such as (i) the intracellular transport/localization of the TBSV replication proteins (16, 17), (ii) the insertion of the p33 into membranes (17), and (iii) the assembly of the tombusviral VRC (18).

Although the viral RdRp activation takes place in solution in our experimental system with the truncated p92-Δ167N RdRp, we have previously shown that the activation of the full-length p92 RdRp protein also requires the presence of cellular membranes (the ER membrane in the CFE assay) and the p33 replication protein in addition to cellular protein factors (11, 41). Similar to the full-length p92, the truncated p92-Δ167N RdRp is also capable of *de novo* RNA synthesis that leads to full-length cRNA on the DI-72 (+)RNA template in the presence of p33 replication protein and subcellular membranes (41). The difference in the activation mechanisms between the p92-Δ167N RdRp and the full-length RdRp is due to the N-terminal portion of the protein, which inhibits RdRp activity until its association with the membrane takes place (41). Nevertheless, the requirements of the Ssa1p Hsp70 chaperone are likely the same for the p92-Δ167N RdRp and the full-length RdRp protein.

Interestingly, the activation of reverse transcriptase for hepadnaviruses depends on Hsp90 proteins (55–57), suggesting that viruses usurp the heat shock protein apparatus to activate viral polymerases. The Hsp70 chaperone family and cochaperones might also be involved in replication of *Dengue virus*, *Hepatitis C virus*, *Red clover necrotic mosaic virus*, *Sindbis virus*, BMV, ToMV, *Turnip mosaic virus*, *Rabies virus*, and *Borna disease virus* (2, 58–67). Hsp70 functions by an ATP hydrolysis-driven conformational change that regulates its substrate binding and release. In addition to being essential for replication of many viruses (1, 2), members of the cytosolic Hsp70 family are involved in many cellular processes, such as the refolding of misfolded or aggregated proteins, the folding of newly synthesized proteins, translocation of organellar and secretory proteins across membranes, insertion of membrane proteins into the membranes, protein complex assembly and disassembly, protein degradation, and receptor signaling (68, 69).

The roles of phospholipids in binding of replication proteins to (+)RNA and RdRp activation. The tombusvirus p33 and p92 replication proteins have to switch from the cytosolic to membranous environment during VRC assembly. How can this dramatic change in the microenvironment affect the biochemical features of these proteins? Here we show that phospholipids, which are the main components of intracellular membranes, affect the activation of the TBSV RdRp and the abilities of the p33 and p92 RdRp proteins to bind to the viral (+)RNA template *in vitro*. Namely, the neutral phospholipids PE and PC, which are the most abundant phospholipids in subcellular membranes, stimulate both activation of the TBSV RdRp and p33-RNA and p92 RdRp-RNA interactions. These effects could be important during tombusvirus replication. This is because PE has been shown to become enriched by p33 at the sites of tombusvirus replication (72). Thus, the viral RdRp becomes activated only when it is present in the suitable PE-rich microenvironment that likely facilitates VRC assembly. The stimulatory effect of PE and PC on p92-RNA interaction should also favor their mutual recruitment to the site of viral replication.

In contrast, the inhibitory effect of PG or CA on p92-RNA interaction and RdRp activation might serve the purpose of preventing tombusvirus RNA synthesis from taking place in a less

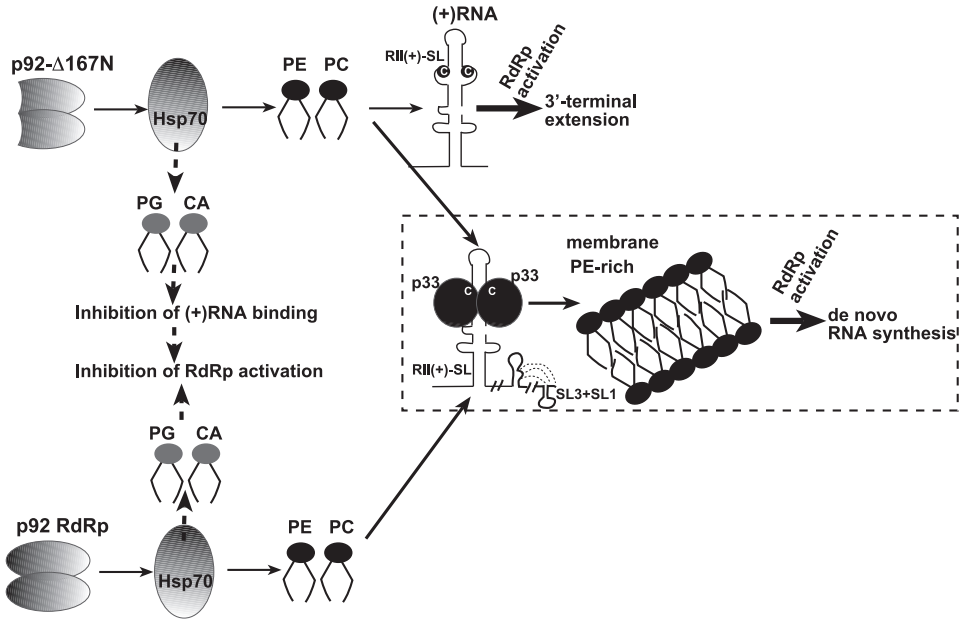

FIG 7 Summary of the effects of Hsp70 and various phospholipids on the TBSV p92-Δ167N RdRp activities. We propose that the p92-Δ167N RdRp protein becomes activated in the presence of Hsp70, PE, or PC and the viral (+)RNA carrying the p33RE *cis*-acting sequence. These components facilitate the 3-TEX activity of p92-Δ167N RdRp *in vitro*. If the p33 replication protein and cellular membrane are also present in the assay (see the area within the dotted box), then p92-Δ167N RdRp could initiate *de novo* and produce full-length complementary product (41). If PG or, to a lesser extent, CA binds to the p92-Δ167N RdRp, then the (+)RNA binding by the RdRp is inhibited, thus blocking the RdRp activation step. Note that the activation of the full-length p92 RdRp (bottom part) is more complex, requiring additional components, such as the p33 replication cofactor and subcellular membranes and additional *cis*-acting elements (SL3+SL1 within the 3′-untranslated region) present in the viral (+)RNA (41).

suitable microenvironment or if mistargeted into the wrong subcellular location. The inhibitory effect of PG on p92-RNA interaction is likely the reason that PG can strongly inhibit the activation of the TBSV RdRp, which requires the p33RE *cis*-acting sequence [consisting of the RII(+)-SL hairpin structure] to become activated (41). Altogether, the complex effects of various phospholipids on different biochemical activities of the TBSV replication proteins seem to serve the need of the virus to replicate only in a favorable microenvironment that is likely well suited for efficient VRC assembly and spherule formation. The favorable microenvironment might help tombusviruses to hide from recognition by the host surveillance system and from destruction by the host innate immunity responses.

These findings might also open up new antiviral approaches based on manipulation of the concentration of inhibitory versus stimulatory phospholipids in host cells. Upregulation of PG seems an especially good target due to the dominant inhibitory effect of PG on TBSV RdRp activation. These possibilities will need to be further tested in *in vitro* replication assays and in cells.

Phospholipids have also been shown to stimulate nodavirus RdRp (protein A) binding to the viral RNA and protein A self-interactions (70, 71). Thus, it is likely that the functions of RdRps of other (+)RNA viruses are also regulated by cellular phospholipids.

A model of the regulatory roles of phospholipids in tombusvirus replication. The different roles of various phospholipids in tombusvirus RdRp activation (i.e., stimulatory roles for PE and PC and dominant inhibitory function for PG) might be utilized by tombusviruses to regulate new VRC assembly during the course of infection (Fig. 7). We propose that the tombusvirus RdRp "measures" the phospholipid homeostasis in infected cells, including the amount of available PE and PC that could be hijacked for viral replication. If enough PE and/or PC is available, then the RdRp could become activated, followed by viral RNA synthesis in the newly assembled spherules. In contrast, if the free/available PE and PC levels are sequestered and not available in the suitable organellar membranes—peroxisomes, endoplasmic reticulum (ER), or mitochondria—in the infected cells, then binding of the tombusvirus RdRp to PG could inhibit RdRp activation. This would block the assembly of the functional VRCs or viral RNA synthesis when the intracellular conditions are no longer optimal for viral replication. Altogether, our model predicts that tombusviruses might be able to sense the status of the phospholipid homeostasis in infected cells in real time via the availability of various phospholipids in the preferred subcellular membranes.

It is important to note here, however, that tombusviruses not only can take advantage of phospholipids present at the beginning of viral infections, but they actually induce *de novo* phospholipid synthesis to render the cells more suitable to support robust tombusviral replication (49). Altogether, tombusviruses likely "make decisions" to assemble or not to assemble new functional VRCs by sensing the phospholipid composition or content of given subcellular membranes. Future experiments will address how tombusviruses rewire the host cell pathways to make membranous microenvironments more favorable for robust viral replication.

ACKNOWLEDGMENTS

We are grateful to Jason Gestwicki (USF) for providing MKT-077 inhibitor. We thank Ching-Kai Chuang for critical comments.

This work was supported by NIH-NIAID (AI109529) and by the University of Kentucky to P.D.N. The mass spectrometric analysis was per-

formed at the University of Kentucky Center for Structural Biology Protein Core Facility. This core facility is supported in part by funds from NIH National Center for Research Resources (NCRR) grant P20 RR020171.

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