Coat Protein Regulation by CK2, CPIP, HSP70, and CHIP Is Required for Potato Virus A Replication and Coat Protein Accumulation

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ABSTRACT We demonstrate here that both coat protein (CP) phosphorylation by protein kinase CK2 and a chaperone system formed by two heat shock proteins, CP-interacting protein (CPIP) and heat shock protein 70 (HSP70), are essential for potato virus A (PVA; genus *Potyvirus*) replication and that all these host proteins have the capacity to contribute to the level of PVA CP accumulation. An E3 ubiquitin ligase called carboxyl terminus Hsc70-interacting protein (CHIP), which may participate in the CPIP-HSP70-mediated CP degradation, is also needed for robust PVA gene expression. Residue Thr²⁴³ within the CK2 consensus sequence of PVA CP was found to be essential for viral replication and to regulate CP protein stability. Substitution of Thr²⁴³ either with a phosphorylation-mimicking Asp (CP^{ADA}) or with a phosphorylation-deficient Ala (CP^{AAA}) residue in CP expressed from viral RNA limited PVA gene expression to the level of nonreplicating PVA. We found that both the CP^{AAA} mutant and CK2 silencing inhibited, whereas CP^{ADA} mutant and overexpression of CK2 increased, PVA translation. From our previous studies, we know that phosphorylation reduces the RNA binding capacity of PVA CP and an excess of CP fully blocks viral RNA translation. Together, these findings suggest that binding by nonphosphorylated PVA CP represses viral RNA translation, involving further CP phosphorylation and CPIP-HSP70 chaperone activities as prerequisites for PVA replication. We propose that this mechanism contributes to shifting potyvirus RNA from translation to replication.

IMPORTANCE Host protein kinase CK2, two host chaperones, CPIP and HSP70, and viral coat protein (CP) phosphorylation at Thr²⁴³ are needed for potato virus A (PVA) replication. Our results show that nonphosphorylated CP blocks viral translation, likely via binding to viral RNA. We propose that this translational block is needed to allow time and space for the formation of potyviral replication complex around the 3' end of viral RNA. Progression into replication involves CP regulation by both CK2 phosphorylation and chaperones CPIP and HSP70.

KEYWORDS E3 ubiquitin ligase CHIP, HSP70, potato virus A, potyvirus, coat protein, heat shock protein HSP40, protein kinase CK2, translation, viral replication

The main function of the capsid and coat proteins (CPs) of positive-strand RNA viruses is to package viral genomes into virions. An increasing body of evidence shows that viral CPs act as important regulators of other viral functions during the infection process as well. CPs modulate translation of viral RNA; binding of a few molecules of alfalfa mosaic virus (AMV) CP to its genomic RNAs is required for efficient translation of viral transcripts (1). The hepatitis C virus core protein modulates translation from the viral 5' internal ribosome entry site (IRES) (2). CPs can also regulate viral RNA replication since, for example, the AMV CP has been shown to enhance RNA synthesis by bridging the viral RdRp and RNA, possibly by inducing a highly ordered

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structure at the 3' end of viral RNA (3, 4). The CP of brome mosaic virus (BMV) enhances synthesis of viral RNA in early infection stages by binding to a 3' end RNA element called SLC present in BMV genomic RNAs. Later in infection, as the level of BMV CP rises, it starts to inhibit both viral replication and RNA translation (5, 6). CPs also regulate the formation of viral replication complexes (VRCs). BMV CP induces membrane rearrangements visually similar to the structures observed in infected cells (7). The CP of tobacco mosaic virus (TMV), although not essential for replication, enhances the formation of VRCs, making them appear sooner, and increases their size (8). In our earlier work, we found that heat shock protein 70 (HSP70) together with its cochaperone CPIP regulates gene expression-associated functions of potato virus A (PVA; genus *Potyvirus*) CP (9). Furthermore, we showed that PVA CP suppresses viral translation via cotranslational CP-CP interactions in a concentration-dependent manner (9, 10).

As the CP often regulates processes during viral infection in a concentration-dependent manner (2, 5), tight control over CP production is required. CP expression from subgenomic RNAs (sgRNAs) can be one way to regulate its levels, allowing the expression of CP in large amounts only after successful replication (11). This mechanism is not used in the case of potyviruses. They exploit a polyprotein expression strategy for CP production. Without additional means of regulation, this strategy results in CP being produced simultaneously and in an equimolar ratio with the replication proteins. How the regulation of CP levels during potyvirus infection is achieved is not yet fully understood, but some mechanisms have been proposed (12). One possibility to down-regulate CP accumulation early in the infection is by directing CP to targeted degradation via the HSP70 and CP-interacting protein (CPIP) chaperone system (9). Another possibility is regulation of CP functions by phosphorylation. Capsid protein phosphorylation has been found to regulate the early events of virus infection and replication in many cases (13–18). For example, point mutations at the phosphorylation sites of cauliflower mosaic virus CP were shown to abolish virus infectivity (15). We have shown that CP phosphorylation by protein kinase CK2 is essential for PVA infection and downregulates CP binding to RNA (19, 20), suggesting that a CP phosphorylation-mediated mechanism controls potyviral CP functions. In the current study, we sought to understand in further mechanistic detail how PVA CP regulates viral gene expression. On the basis of the results obtained, we propose that PVA CP regulation by CK2, HSP70, and CPIP is an essential component of PVA infection, possibly shifting PVA RNA from translation to replication.

RESULTS

The CK2 phosphorylation site in CP is important for PVA replication. Our earlier study showed that mutations both mimicking and prohibiting CK2-mediated phosphorylation of CP impair the development of systemic PVA infection (19). The preferred site for the *in vitro* phosphorylation of PVA CP by protein kinase CK2 is Thr²⁴² at the CK2 consensus site ²⁴²TTSEED²⁴⁷ (19). However, both the threonine residues and the serine residue within this site fulfill the criteria of being a CK2 phosphorylation site. When they were all mutated to alanine residues (CP^{AAA}), the virus did not move either from cell to cell or systemically. We could nevertheless detect a CP^{ATA} reversion rendering the virus capable of systemic infection at 20 days postinfection (dpi) (19), showing that Thr²⁴³ alone is essential for PVA infection.

As a next step in characterizing the importance of CP regulation by CK2, we aimed at distinguishing whether disruption of this mechanism causes the single-cell infection phenotype due to impaired replication or cell-to-cell movement. For this, we introduced mutations into the CK2 phosphorylation consensus site-encoding sequence in the *Rluc*-containing PVA infectious cDNA (icDNA) and quantified viral gene expression. The introduced mutations were ²⁴²AAAEED²⁴⁷ (phosphorylation-deficient PVA^{AAA}), ²⁴²ADAEED²⁴⁷ (phosphorylation-mimicking PVA^{ADA}), ²⁴²ATAEED²⁴⁷ (natural revertant PVA^{ATA}), and finally a fourth one with nucleotide substitutions affecting the encoding RNA but not the ²⁴²TTSEED²⁴⁷ protein sequence (PVA^{silent}). Gene expression levels from PVA^{AAA}, PVA^{ADA}, PVA^{ATA}, and PVA^{silent} were compared to that of the

replication-deficient PVA^{ΔGDD}, the movement- and assembly-deficient PVA^{CPmut} virus, and wild-type PVA (PVA^{WT}). All these constructs are schematically presented in Fig. 1A. When these PVA iDNAs were expressed in *Nicotiana benthamiana* plants via *Agrobacterium* infiltration, we found equal expression levels of PVA^{silent}, PVA^{ATA}, and PVA^{WT}. This reinforced that Thr²⁴³, not Thr²⁴², is the essential residue to be phosphorylated during PVA infection *in planta*. Gene expressions of both the phosphorylation-mimicking PVA^{ADA} and the phosphorylation-deficient mutant PVA^{AAA} stayed at the level of nonreplicating PVA^{ΔGDD} and significantly below PVA^{CPmut} expression levels (Fig. 1B), which suggests that they have primary defects in replication rather than cell-to-cell movement. Interestingly, PVA^{ADA} expression level is higher whereas PVA^{AAA} expression is lower than that of PVA^{ΔGDD} in a statistically significant manner (Fig. 1C). These data suggest that CK2-mediated phosphorylation of CP is important for PVA replication and that PVA^{AAA} RNA is defective in translation whereas PVA^{ADA} RNA is translated more efficiently than PVA^{ΔGDD}. A comparison of 18 potyviral CP sequences indicates that the CK2 consensus sequence and either Thr or Ser at the position corresponding to the Thr²⁴³ of PVA CP are fully conserved among these viruses (Fig. 1D).

CK2 is found in membrane-associated viral RNP complexes. In our earlier work, we identified HSP70 and CPIP as components of a membrane-associated viral RNP complex in PVA-infected plants with functions in regulating CP to support replication (9). In that study, we identified HSP70 and CPIP as crucial components of viral translation associated with potyvirus replication. As the results presented above suggested that CP is regulated by CK2 and this is essential for replication, we analyzed whether the CK2 α -subunit was associated with these viral complexes. For this purpose, PVA^{NlbSIII} and PVA^{VPgSIII} iDNAs and Strep-tag purification were used, as described in reference 9, to purify viral replication proteins VPg and Nlb from membrane fractions of infected plants. PVA^{NlbSIII} and PVA^{VPgSIII} iDNAs infect plants similarly to PVA^{WT} as judged by the expression of virus-encoded green fluorescent protein (GFP) (9). Western blot analysis revealed specific copurification of CK2 α -subunit with VPg and Nlb from the membranes that harbor potyviral replication complexes and polysomes on which active potyviral RNA translation occurs (Fig. 2) (21–24). These results place CK2 in close association with PVA replication proteins, which is consistent with the hypothesis that CK2 phosphorylates CP in connection to PVA translation and/or replication.

CPIP, HSP70, CK2, CHIP, and the proteasome regulate CP accumulation. Expression of a dominant negative CPIP mutant (CPIP^{Δ66}) leads to strong accumulation of CP in the absence of PVA infection (9). CPIP^{Δ66} lacks the J domain through which native CPIP recruits HSP70 to promote CP ubiquitination and degradation. The carboxyl terminus Hsc70 interacting protein (CHIP) is an established E3 ubiquitin ligase that interacts with HSP70 and HSP90 to catalyze polyubiquitination of their target proteins (see references 25 and 26 and references therein). We therefore regarded CHIP as the most likely candidate E3 ubiquitin ligase for HSP70-CPIP-driven CP ubiquitination. To test whether CK2, HSP70, or CHIP can influence CP accumulation, we coexpressed CP^{WT} with RNA hairpin constructs that initiate CK2, HSP70, and CHIP silencing. After 4 days, enzyme-linked immunosorbent assay (ELISA) and Western blot analyses showed that silencing of these host factors led to an approximately 2-fold increase in CP accumulation, suggesting that CK2, HSP70, and CHIP promote CP degradation (Fig. 3A and B). The origin of the double band in Fig. 3B is not clear, although it is possible to conclude that the two forms are not due to phosphorylation of the CK2 site, as also CP^{AAA} migrates similarly. Fluc was also coexpressed in the analysis to function as an internal control, and its unaltered levels underscore that increased protein accumulation was CP specific (Fig. 3C). We also examined in the CK2-, HSP70-, and CHIP-silenced background the level of CP mRNA accumulation. While CK2 and HSP70 silencing did not alter CP mRNA amounts in comparison to that of the empty silencing vector (hp–) used as a control, CHIP silencing was found to do so (Fig. 3F). Therefore, the role of CHIP remains elusive in CP turnover, as an increased CP mRNA amount likely accounts for the

A Plasmid Construct backbone name

Construct details

B

C

D

Potato_Virus_A/227-264
 Wild_tomato_mosaic_virus/703-740
 Pepper_veinal_mottle_virus_gi|972821509|dbj|BAU24765.1|/532-569
 Chilli_veinal_mottle_virus_gi|146334975|gb|ABQ23275.1|/455-492
 Japanese_yam_mosaic_virus_gi|932510666|gb|ALG64834.1|/453-490
 Plum_pox_virus_gi|224174819|emb|CAX51129.1|/493-530
 Chilli_ringspot_virus_gi|386656523|gb|AFJ19354.1|/420-457
 Tobacco_vein_banding_mosaic_virus_gi|157886726|emb|CAJ87153.1|/437-474
 Tulip_breaking_virus_gi|545669888|gb|AGW45381.1|/421-458
 Narcissus_yellow_stripe_virus_gi|28301539|emb|CAC85379.1|/443-480
 Ranunculus_mild_mosaic_virus_gi|129593894|gb|ABO31118.1|/430-467
 Jasmine_yellow_mosaic_potyvirus_gi|100309537|gb|AMO02836.1|/444-481
 Kalanchoe_mosaic_virus_gi|259157139|gb|ACV96786.1|/262-299
 Narcissus_late_season_yellows_virus_gi|28301573|emb|CAD38487.1|/443-480
 Carrot_thin_leaf_virus_gi|8895432|gb|AAF80962.1|/AF203530_1/440-477
 Turnip_mosaic_virus_gi|118638552|gb|ABL09435.1|/446-483
 Delphinium_vein-clearing_virus_gi|209972496|gb|ACJ03756.1|/439-476
 Jasmine_ringspot_virus_gi|985485913|ref|YP_009221992.1|/237-274

230 235 240 245 250 255 260

L KNSNTNMFGL DGNV **TTSEED** TERHTATDVNRNMHHL

L RNANRNMFG L DGKV **GTQEED** TERHTAEDVNRNMHNL

L RNANRNMFG L DGKV **GTQEED** TERHTAEDVNRNMHNL

L RGVSNRMFG L DGRV **GTQEED** TERHTAEDVNRNMHNL

L RGVQSKL FGL DGNV **STMEENT** TERHTAEDVNRNMHSL

L RNVQNR L FGL DGNV **GTQEED** TERHTAGDVNRNMHNL

VMHAKNNMFG L DGNV **STKEENT** TERHTATDVNRDMHHL

VANSKNNL FGL DGNV **STKEENT** TERHTATDVNRNMHHL

L RNSNNKL FGL DGNV **STQGED** TERHTTDDVNGNMHTL

L RGAKNHL FGL DGNV **GSADENT** TERHTTDDVNRNMHNL

L RNANTRMFG L DGNV **STQAED** TERHTTDDVNRNMHTM

L RNASR L FGL DGNV **GTKEED** TERHTADDVNRNMHNL

L RSAKNNL FGL DGNV **GTTEENT** TERHTTDDVNRNMHSL

L RGTQNH L FGL DGNV **GTTEENT** TERHTTDDVNRNMHSL

L RNASNKL FGI DGKV **SQEED** TERHTTDDVNRNMHSM

L RGANNNL FGL DGNV **GTTVENT** TERHTTDDVNRNMHNL

VRNAKNMFG L DGNV **GTSEED** TERHTANDVSTKMHS L

L RNASR L FGL DGNV **GTKEED** TERHTADDVNRNMHNL

FIG 1 The CK2 phosphorylation consensus sequence is necessary for PVA replication. (A) Full-length PVA icDNA constructs. The amino acid mutations in the CK2 phosphorylation consensus region are shown under the CP coding sequence, and the nucleotide sequence corresponding to the mutated region is shown on the left. (B) Plants were infiltrated with various PVA icDNA constructs carrying *Renilla* luciferase gene as a reporter. *Renilla* luciferase activity values derived from PVA mutants were measured at 3 dpi. (C) *Renilla* luciferase activity values were measured at 3 dpi from the nonreplicating PVA mutant PVA^{ΔGDD} and two CK2 consensus sequence mutants, PVA^{AAA} and PVA^{ADA} ($n = 9$). (D) Alignment of 18 potyviral CP sequences showing the CK2 phosphorylation consensus site (boxed area). Conservation of either Thr or Ser residue at the location corresponding to T²⁴³ of PVA CP is marked in bold. Amino acid numbering is according to the PVA sequence. **, $P < 0.01$.

FIG 2 CK2 α-subunit was copurified with Nib and VPg from a crude membrane fraction. Western blot analysis of samples pulled down with StrepIII-tagged PVA protein Nib (Nib^{SIII}) or VPg (VPg^{SIII}) from the membrane fractions probed with anti-CK2 α antibody shows that protein kinase CK2 α-subunit is copurified with PVA replication proteins.

increased CP amounts to some extent. Silencing of the host proteins was confirmed either by reverse transcription-quantitative PCR (RT-qPCR) or by Western blotting (Fig. 3G and H). However, inhibition of the proteasome by MG132 resulted in a 2-fold CP accumulation similar to that seen with silencing CK2, CHIP, and HSP70 (Fig. 3D). These

FIG 3 Regulation of CP accumulation by CK2, HSP70, CHIP, proteasome, and CK2 phosphorylation site within CP. (A and B) The host factors CK2, CHIP, and HSP70 were transiently silenced by RNA hairpin constructs, and the effect on the accumulation of CP^{WT} was measured in a noninfection context as shown by ELISA-based quantification (A) and Western blotting with anti-CP antibody (B) at 4 dpi. CP^{WT} was *Agrobacterium* infiltrated at an OD₆₀₀ of 0.5, and silencing constructs were infiltrated at an OD₆₀₀ of 0.3. (C) Transiently expressed Fluc enzymatic activity was not affected by the silencing of CK2, CHIP, and HSP70. (D) The 26S proteasome activity was inhibited with MG132 compound, and the accumulation of CP^{WT} was measured by ELISA in a noninfection context. (E) Accumulation of PVA CP phosphorylation-deficient and phosphorylation-mimicking mutants is increased compared to that of CP^{WT} at 3 dpi. (F) RT-qPCR was used to quantitate the mRNA levels of transiently expressed PVA CP in CK2, CPIP, HSP70, and CHIP silencing backgrounds at 4 dpi. (G and H) RT-qPCR of CPIP and CHIP mRNA levels (G) and Western blotting with anti-CK2 and anti-HSP70 antibodies (H) were used to verify silencing at 4 dpi after initiation of silencing. hp- is an empty silencing vector used as a control. The following significance code is used: *, $P < 0.05$; **, $P < 0.01$.

FIG 4 CPIP is able to regulate only CP^{WT} and CP^{AAA} but not CP^{ADA} levels, although all CP variants bind CPIP. The accumulation of CP^{WT} (A), the phosphorylation-deficient mutant CP^{AAA} (B), and the phosphorylation-mimicking mutant CP^{ADA} (C) was monitored by ELISA and Western blotting when coexpressed with CPIP or CPIP^{Δ66} or during CPIP silencing (hpCPIP) at 4 dpi. The following significance codes are used: **, $P < 0.01$; *, $P < 0.05$; nd, not detectable. (D) CPIP^{Δ66} or GUS as a control was coexpressed with PVA CP mutants in a noninfection context. CPIP^{Δ66} was immunoprecipitated at 4 dpi, and the eluates were probed for the presence of PVA CP by Western blotting (top [pd]). The total amounts (prior to purification) of CP are shown in the second panel (in). The presence of CPIP^{Δ66} was verified by probing with α -myc antibody and an equal amount of antibodies with anti-mouse antibody and loading by staining the membranes with Ponceau S solution. CPs were infiltrated at an OD₆₀₀ of 0.5 and all other constructs at an OD₆₀₀ of 0.3.

results support the involvement of CK2, the proteasome, and putatively CHIP in regulation of PVA CP in addition to the previously established CPIP and HSP70 (9).

As silencing of CK2 caused CP accumulation, a logical interpretation was that the phosphorylation status of the CK2 consensus site in CP affects its turnover. Therefore, we studied the accumulation of CPs mutated in the CK2 site by Western blotting (Fig. 3E). Both the phosphorylation-deficient (CP^{AAA}) and phosphorylation mimic (CP^{ADA}) mutants accumulated to higher levels than CP^{WT}. Accumulation of both CP^{AAA} and CP^{ADA} compared to that of CP^{WT} suggested that the lack of dynamic phosphorylation reduces CP turnover.

CPIP contributes to the suppression of the accumulation of CP^{WT} and CP^{AAA}, but not CP^{ADA}. We next analyzed how overexpression of CPIP or CPIP^{Δ66} affected CP^{WT}, CP^{AAA}, and CP^{ADA} accumulation levels. The RNA hairpin construct targeting CPIP was also included in the experiment. As expected on the basis of our earlier results (9), the overexpression of CPIP reduced the CP^{WT} amount well below the control level and rendered it close to undetectable (Fig. 4A). Although hairpin-mediated silencing is not

complete and some endogenous CPIP may have remained as in Fig. 3G, accumulation of CP^{WT} increased in the presence of hpCPIP compared to the controls. Still, a substantially higher accumulation of CP^{WT} was observed when the dominant negative mutant CPIP^{Δ66} was overexpressed. The phosphorylation-deficient mutant CP^{AAA} behaved similarly to CP^{WT} in its response to CPIP silencing and CPIP^{Δ66} overexpression (Fig. 4B). However, the phosphorylation-mimicking mutant CP^{ADA} did not respond to CPIP, as it accumulated to high levels under all the tested conditions (Fig. 4C). These data suggest that phosphorylated CP may escape degradation via the CPIP pathway, whereas the nonphosphorylated CP is capable to enter the pathway provided that CPIP is present in sufficient amounts.

CP phosphorylation prevents CP degradation downstream of CPIP. To understand further the link between CK2-mediated phosphorylation of CP and CPIP-mediated regulation of CP functions, we wanted to find out whether CK2 phosphorylation plays a role in the CP-CPIP interaction. The dominant negative J-domain mutant CPIP^{Δ66} still binds potyviral CP (27). Because CPIP functions as a cochaperone of HSP70 via interaction of the J domain (28), the CPIP^{Δ66} mutant is unable to deliver CP to HSP70 and the degradation pathway. Therefore, to allow the formation of a more stable and detectable CPIP-CP complex, we coexpressed PVA CP and the phosphorylation site mutants with a myc-tagged CPIP^{Δ66}. This was followed by immunoprecipitation of CPIP^{Δ66}, and subsequent detection of CPs showed that CP^{WT}, CP^{AAA}, and CP^{ADA} all bind CPIP (Fig. 4D). This result showed that CK2-mediated phosphorylation of Thr²⁴³ in CP is not regulating binding of CPIP to CP.

The phosphomimic CP^{ADA} does not, whereas the phosphorylation deficient CP^{AAA} does, inhibit PVA translation. When CP^{WT} is present in high concentrations, it inhibits PVA RNA translation (9, 10). The results reported in reference 10 suggest that the inhibition occurs through cotranslational interactions between CP accumulated *in trans* and CP translated from viral RNA *in cis*. Our next question was whether CP^{AAA} and CP^{ADA} have the capacity to suppress PVA translation when expressed *in trans*. CP^{WT}, which strongly inhibits, and CP^{mut}, which does not inhibit, PVA gene expression were used as controls for comparison. To this end, PVA^{WT} and PVA^{ΔGDD} were coexpressed together with different CPs, and viral gene expression was determined by RLuc activity at 3 dpi (Fig. 5). CP^{AAA} inhibited PVA gene expression as strongly as the CP^{WT}, resulting in almost complete inhibition of PVA translation. However, similarly to CP^{mut}, CP^{ADA} had only a low inhibitory effect on PVA translation. The effect of CPs on PVA^{ΔGDD} gene expression was similar to the effect on PVA^{WT} gene expression (compare left and right panels in Fig. 5), indicating that the inhibition happens at the translational level. Our conclusion is that the capacity of CP to inhibit PVA translation can be downregulated via phosphorylation of Thr²⁴³ of CP. In support of this, CK2 overexpression together with nonreplicating PVA led to significantly increased translation whereas CK2 silencing led to significantly reduced translation of viral RNA (Fig. 5). PVA^{WT} gene expression was also increased by CK2 overexpression (Fig. 5), indicating that CK2 promotes PVA infection, possibly by increasing viral translation.

Host proteins CK2, CPIP, HSP70, and CHIP are all required to achieve a normal level of gene expression during PVA infection. Our results show that mutations altering the CP CK2 phosphorylation site cause defects in PVA replication, that CK2, CPIP, HSP70, and CHIP regulate CP accumulation and that phosphorylation of Thr²⁴³ regulates the ability of CP to inhibit PVA translation. Altogether, we hypothesized that CK2, CPIP, HSP70, and CHIP are all involved in regulating the CP primarily during translation/replication rather than particle assembly and virus cell-to-cell movement. To test whether the effect on viral gene expression is related to viral movement, we set up an experiment in which PVA-GFP was infiltrated at a low *Agrobacterium* concentration (optical density at 600 nm [OD₆₀₀], 0.0005) during transient silencing of the host proteins CPIP, CHIP, CK2, and HSP70. Accordingly, the low concentration of *Agrobacterium* carrying PVA-GFP icDNA allowed for visualization of individual infection foci. The silencing was initiated with hairpin constructs 1 day before infiltration of virus icDNA to

FIG 5 PVA RNA translation is regulated by CP in a CK2 phosphorylation-dependent manner. The gene expression, reported by *Renilla* luciferase activity, of wild-type PVA (PVA^{WT}) (left) and replication-deficient PVA (PVA^{ΔGDD}) (right) was measured at 3 dpi in the context of overexpressing the wild-type PVA CP (CP^{WT}), nonphosphorylatable CP (CP^{AAA}), and phosphorylation-mimicking CP (CP^{ADA}). As a control, a CP mutant debilitated in viral RNA binding was used (CP^{mut}) (leftmost bar charts on both panels). Additionally, the gene expression of PVA^{WT} was measured during overexpression of tCK2α (PVA^{WT}, right bar chart) and the gene expression of PVA^{ΔGDD} during overexpression of tCK2α and silencing of CK2α (PVA^{ΔGDD}, middle and rightmost bar charts, respectively) at 3 dpi. Anti-CK2α immunoblots indicate the level of CK2α when overexpressed and silenced as a comparison to the control samples.

ensure strong silencing when evaluating possible effects on cell-to-cell movement. PVA infection focus sizes and their amount were measured at 3 days after viral icDNA infiltration. Despite the size of infection foci being somewhat variable within treatments, no significant difference in focus size appeared between the treatments (Fig. 6A). There was also no difference in the number of initiated infections between the different treatments (Fig. 6B). Moreover, PVA gene expression and RNA amount was significantly lower due to the silencing (Fig. 6C and D). Representative figures showing infection foci in various silencing backgrounds are presented in Fig. 6E.

These data suggest that silencing of CK2, CPIP, HSP70, and CHIP does not impair cell-to-cell movement but instead reduces the level of PVA gene expression at the single-cell level. This shared phenotype of the host factors supports their importance in regulating CP during replication-associated translation of PVA.

DISCUSSION

The conventional role of the potyviral CP is to encapsidate the viral genome in particles and aid in transportation of the virus within and between plants. However, as shown for several plant viruses, CPs can have many more functions during infection (reviewed in references 12, 29, and 30). These include CP-mediated regulation of viral RNA replication and translation, for which, e.g., brome mosaic virus represents a well-studied example (see reference 31 and references therein). In the present study, we aimed to deepen our understanding of how CP regulates potyvirus infection. Our previous studies have established that the phosphorylation of CP by CK2 has an essential role in PVA infection, the importance of which could be in regulating the interaction between CP and viral RNA (19, 20). We have additionally found that the host chaperones CPIP and HSP70 are important factors of potyvirus replication and mediate CP degradation (9). Degradation of CP by the CPIP/HSP70 pathway is important to maintain replication and thereby enables much stronger infection by potyviruses (9, 27). In addition, we have established that CP inhibits viral gene expression via cotranslational interactions between CPs accumulated in *trans* and CP produced from PVA RNA in *cis* (10). The current study revealed that similarly to CPIP and HSP70, both CK2 and the CK2 phosphorylation site within PVA CP are important for PVA replication and that

FIG 6 Host factors CK2, CPIP, CHIP, and HSP70 are required for robust PVA expression at a single-cell level. The host factors CK2, CPIP, CHIP, and HSP70 were transiently silenced by RNA hairpin constructs, and the effects on the size of infection foci (A) and the infection initiation efficiency of PVA^{GFP} (B) were measured. (C and D) PVA gene expression (C) and RNA levels (D) were measured in these silencing backgrounds. RNA hairpin (hp) silencing constructs were infiltrated at an OD₆₀₀ of 0.3 1 day before the virus, which was infiltrated at an OD₆₀₀ of 0.05 for PVA^{WT} and an OD₆₀₀ of 0.0005 for PVA^{GFP}. Viral movement and gene expression were measured at 3 dpi. (E) Representative images of PVA-GFP infection foci in the CK2, CPIP, HSP70, and CHIP silencing backgrounds at 3 dpi. Asterisks denote statistical significance: **, $P < 0.01$; *, $P < 0.05$.

CK2 together with CPIP, HSP70, and putatively CHIP regulate CP accumulation. In addition, we found that CP phosphorylation by CK2 regulates PVA translation, and this is likely linked to the different RNA binding capacities of the nonphosphorylated and phosphorylated CP. Our interpretation of the results is represented by a hypothetical model for discussion (Fig. 7).

Our earlier studies have established that CK2-mediated phosphorylation of CP is important for PVA infection, as CP phosphorylation site mutants failed to spread from cell to cell (19). However, it remained unclear whether this defect was at the single-cell level or movement related. In the present study, we found that CP-phosphorylation mutants had gene expression levels comparable to those of the nonreplicating PVA^{AGDD} rather than nonmoving PVA^{CPmut}, suggesting that CK2-mediated CP phosphorylation is important for replication. Moreover, because replication is impaired for both the phosphomimetic (PVA^{ADA}) and the phosphodeficient (PVA^{AAA}) mutants, dynamic regulation between the two forms of CP appears to be important in the process.

The CK2 phosphorylation consensus site resides at a putative RNA binding domain according to a PVA CP structural model (32). In agreement with this, CK2 affects the capacity of CP to bind RNA (20) and altogether supports that the RNA binding capacity of potyviral CP is needed for replication. A silent CK2 site mutant, in which only the RNA sequence was changed, as well as PVA^{ATA}, carrying T242A and S244A mutations in CP, replicated to levels comparable with those of PVA^{WT} and caused full infection. This

FIG 7 Proposed model of CP-regulated shift from translation to PVA replication. The nonphosphorylated CP binds to viral RNA and blocks translation. The translational block by CP enables removal of ribosomes and recruiting of Nlb to the 3' UTR of PVA RNA for the assembly of the viral replication complex (VRC). CK2-mediated phosphorylation of CP assists CPIP and HSP70 in detaching CP from viral RNA, and E3 ubiquitin ligase CHIP is recruited by HSP70 for CP ubiquitination and degradation. Removal of CP from PVA RNA allows initiation of the replication reactions.

supports that the CK2 consensus site of PVA CP and PVA replication have a link at the protein level rather than at the encoding RNA level. Conservation of the CK2 consensus site in potyviruses also suggests a functional importance for CP at the protein level. For comparison, it is interesting that tobacco etch virus (TEV) replication tolerates large deletions in its CP (33). More specifically, it was established that a CP protein fragment located after residue 189 needs to be translated for successful replication. At the same time, viruses carrying C-terminal CP deletions were functional in replication only if the deletion started after residue 245. Intriguingly, the putative CK2 phosphorylation site of TEV CP (²³⁶VG**T**AEEED²⁴², where boldface type indicates the Thr conservation site) lies within the peptide needed for replication (33). However, a *cis*-active RNA element was proposed to overlap the minimal essential TEV CP protein fragment and it was also proposed to be required for TEV replication.

In our previous study (10), we proposed that the site for the CP-RNA interaction on viral RNA is at the CP-encoding region and the binding to this site occurs in the context of ongoing translation. To initiate potyviral replication complex assembly, 6K2 tethers Nla to the endoplasmic reticulum (ER) membranes while Nlb is directed via the Nla interaction and by the secondary structures in the 3' untranslated region (UTR) and the 3' part of the CP-encoding region to the 3' end of the viral RNA (34, 35). It has been shown that the RNA polymerase, Nlb, of tobacco vein mottling virus (TVMV; genus *Potyvirus*) interacts with CP (36). Interestingly, this interaction was weakened in a catalytically inactive Nlb mutant. We found that CK2 α associates with the VPg- and Nlb-containing protein complexes purified from the crude cellular membranes and showed previously that the similarly purified complexes also contain HSP70, CPIP, viral RNA, and CP (9). On the basis of these data, it is possible that CP interacts directly with

Nlb on viral RNA or that CP and VPg/Nlb are interconnected via viral RNA. Collectively, these results place CK2 and CP in association with the replication complex consisting of Nlb and Nla and reinforce the link between CP phosphorylation and PVA replication. When PVA replication complexes were purified via the membrane-spanning 6K2, only a minor amount of CP compared to that of replication proteins Nla and Nlb was found in these membranous structures (24). This would speak for a transient presence of CP on the viral RNA to be replicated whereas it may not be required for the replication reactions *per se*.

CPiP is an important regulator of potyviral CP required for replication and acts via binding CP and delivering it to HSP70. This is likely followed by CP ubiquitination and degradation and is essential for potyviruses to avoid premature shutdown of replication (9, 27). CHIP is the anticipated E3 ubiquitin ligase to associate with HSP70 and mediate polyubiquitination of CP to promote its degradation by the proteasome (37, 38). CHIP downregulation affected CP mRNA accumulation to some extent, and therefore the role of CHIP in CP degradation remains still putative. Proteasome inhibition caused CP accumulation. Similarly to HSP70, CPIP, and CK2, we also established that CHIP was important for a robust PVA infection unrelated to intercellular movement. Based on our previous study linking CPIP and HSP70 to CP regulation (9), we now propose that both CHIP and CK2 are likewise involved in regulating CP during PVA RNA translation and replication cycle and not in cell-to-cell movement.

Global proteomic analyses of protein ubiquitination show that phosphorylatable amino acid residues flank ubiquitinated Lys residues with high propensity (39, 40). Protein phosphorylation has been linked both to activation (41) and to inhibition (42, 43) of proteasome-mediated degradation of the target protein. One of the questions of the current study was to address if CK2-mediated phosphorylation affects degradation of CP via the CPIP pathway. The CK2 phosphorylation-mimicking mutant CP^{ADA} can bind CPIP but did not undergo CPIP-mediated degradation, suggesting that CP phosphorylation could protect from degradation. However, our data also support that the interactions between CK2, CPIP, HSP70, and CP may occur on viral RNA, and therefore it is possible that CP^{ADA} does not localize to a context where the necessary interactions to enter the degradation pathway take place. We do not exclude the alternative or even parallel possibility that CPs are phosphorylated prior to binding to viral RNA. In such case, it is feasible to speculate that phosphorylation could promote sufficient CP accumulation by protecting it from degradation and simultaneously keep a certain proportion of CP away from inhibiting viral RNA. Production of CP^{AAA} in turn may lead to a situation where most CP molecules bind viral RNA. In the absence of CP phosphorylation, the CPIP/HSP70 system either is dysfunctional or gets overpowered sooner than in the presence of CP^{WT}. In our previous work (19), we have shown that CP^{AAA} produced in *Escherichia coli* is able to form viruslike particles (VLPs). The basis for the stability of the CP^{AAA} mutant when produced from monocistronic mRNA could therefore be the formation of VLPs via CP-CP interactions. To this end, a conceivable role of CP^{WT} phosphorylation would be to aid CPIP and HSP70 functions by downregulating either the RNA binding affinity or the proportion of CP molecules binding to RNA.

Although many important questions remain, in conclusion of this study a plausible scenario could be that binding of nonphosphorylated CP to viral RNA in the vicinity of the 3' UTR of PVA RNA keeps that region free from ribosomes and allows time for the assembly of the replication complex. CP phosphorylation and the CPIP and HSP70 chaperone system remove CP from PVA RNA and open viral RNA for the replication reactions.

MATERIALS AND METHODS

Plants and virus and plant expression constructs. *Nicotiana benthamiana* plants were kept under greenhouse conditions and infiltrated with *Agrobacterium tumefaciens* strain C58C1 pGV2260 at the four- to six-leaf stage. Viral constructs and viral protein expression constructs used in this study were based on the full-length infectious cDNA (icDNA) copies of PVA strain B11 (GenBank accession number AJ296311).

icDNA constructs for PVA^{WT}, PVA-GFP, PVA^{CPmut}, PVA^{AGDD}, PVA^{AAA}, PVA^{NibSIII}, and PVA^{VPgSIII} are described in references 9 and 19. icDNA constructs for PVA^{ATA}, PVA^{ADA}, and PVA^{silent} were done using site-directed mutagenesis using primers CP-ATA-Fwd (5' GCA ACT GCA GAA GAG GAC ACA GAA AG 3') for CP^{ATA}, CP-ADA-Fwd (5' GCA GAT GCA GAA GAG GAC ACA GAA AG 3') for CP^{ADA}, and CP-silent-Fwd (5' ACG ACG AGC GAA GAG GAC ACA GAA AG 3') for CP^{silent} to modify the plasmid pKJE4 carrying the PVA CP-coding region. From pKJE4, the mutated CP constructs were cut out with restriction enzymes MluI and AgeI and inserted into pKJE23 using the same restriction enzyme sites to replace the CP in PVA icDNA. The mutated PVA icDNA was transferred to the binary agro vector pRD400 using KpnI and SalI. The final icDNA constructs in pRD400 are under the control of CaMV 35S promoter and *A. tumefaciens* nopaline synthase transcriptional terminator (*nos*).

Transient expression constructs for individual CPs were done as follows: CP-coding regions were amplified from their respective icDNA using primers CP_N-termATG_F (5' CCG GTA CCA TGG CCG AAA CTC TTG ATG CAA G 3') and CP_C-term_R (5' GGT CTA GAT TAC ACC CCC TTC ACG CCT A 3') and Phusion DNA polymerase (Thermo Scientific). The forward primer adds a KpnI restriction site and an in-frame ATG codon, and the reverse primer adds an XbaI restriction site directly after the TAA stop codon. The CP PCR fragments were digested with KpnI and XbaI restriction enzymes and inserted into the same sites in pHTT690, which contains CaMV 35S promoter and *nos* terminator. From pHTT690, the 35S-CP-*nos* fragments were transferred to the binary agro vector pRD400 using HindIII restriction sites.

To produce the transient expression construct of the tCK2 α , the tCK2 α gene was amplified by PCR from pQE30::tCK2 (19) using primers 5' ATC GCT CGA GAT GTC AAA AGC TCG TGT TTA CA 3' and 5' CGA TGG ATC CTT ACT GCG TCC TCA TCC T 3' carrying XhoI and BamHI restriction sites, respectively. After XhoI and BamHI restriction, the PCR fragment was inserted into the same restriction sites in the multicloning site of pRT101, providing CaMV 35S promoter and terminator. From pRT101, the 35S-tCK2-T-35S cassette was cleaved out with HindIII and inserted into pRD400 binary vector.

To make hairpin silencing constructs targeting CK2, CPIP, CHIP, or HSP70, around 300- to 400-bp fragments of these genes were PCR amplified from *N. benthamiana* cDNA. The PCR fragments were inserted into pDONR^{ZE0} plasmid and finally to pHellsGate8 plasmid using the Gateway cloning system. To amplify CK2, primers CK2sil-Fwd (5' CAG AAC CTC TGT GGG GGA CCA AC 3') and CK2sil-Rev (5' CAT TCC TGC AAA CAT GCA TCC AAG 3'), for CPIP, primers CPIPsil-Fwd (5' AGT GAA GAA GAT TTG AAA AAA TCG 3') and CPIPsil-Rev (5' TTT GTG CCT TTC TTC CAA C 3'), for CHIP, CHIPsil-Fwd (5' CTG CCA TTG ATG CTT ATA CCG AGG C 3') and CHIPsil-Rev (5' TTT GCT TTA GCA AGC TCT TCC CT 3'), and for HSP70, primers HSP70sil-Fwd (5' CCG GAT CCA TGG CTG TCA AAG GAG AAG 3') and HSP70sil-Rev (5' CCC GAT CGG CAT GCT TAG TCG ACT TCC TCA ATC 3') were used.

DAS-ELISA. For double-antibody sandwich ELISA (DAS-ELISA), samples were collected 4 days after infiltrating *N. benthamiana* plants with *Agrobacterium* carrying protein expression constructs. The *Agrobacterium* concentrations used for infiltration are given in the figure legends. Infiltrated plant tissue was homogenized in sampling buffer (Tris-buffered saline [TBS], 0.5% Tween 20, 2% polyvinyl pyrrolidone, and 0.2% bovine serum albumin [BSA], pH 7.4) at a ratio of 1 g of plant material per 3 ml of buffer and centrifuged at $1,000 \times g$ for 5 min to remove cell debris. The undiluted supernatant was used in subsequent steps. Ninety-six-well plates (Costar 3590, high binding; Corning Incorporated, USA) were coated with PVA CP-specific monoclonal antibody (1041-07 PVA, 58/0, MAB; Adgen Phytodiagnostics) using a 1:1,000 dilution in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) and 100 μ l of buffer per well and incubated at 37°C for 3 h. After washing the plate 3 times with 300 μ l of washing buffer (50 mM Tris-Cl, 150 mM NaCl, 0.5% Tween 20 [pH 7.4]) per well, 100 μ l of sample supernatant per well was added and the plate was incubated overnight at 4°C. The next day, the plate was washed as previously and 100 μ l per well of 1:4,000 diluted alkaline phosphatase-conjugated anti-PVA-CP antibody (1041-07 PVA, 58/0, MAB-AP; Adgen Phytodiagnostics) was added, and the plate was incubated at 37°C for 3 h. After another wash, detection of CP was done by using 0.5 mg/ml phosphatase substrate (5-mg tablets; Sigma-Aldrich) in substrate buffer (0.1 M diethanolamine [pH 9.8]), 100 μ l per well. Absorbance was recorded with the Bio-Rad 680 microplate reader at 405 nm. Different concentrations of heterologously produced PVA CP were used as protein concentration standards.

Immunoprecipitation of CPIP^{Δ66}. Samples were collected 4 days after infiltrating *N. benthamiana* plants with *Agrobacterium* containing protein expression constructs (CPs at an OD₆₀₀ of 0.5, GUS and CPIP^{Δ66} at an OD₆₀₀ of 0.3). Lysates were prepared using 3 ml sampling buffer (TBS, 0.5% Tween 20, 1 mM phenylmethylsulfonyl fluoride [PMSF]) per 1 g of plant tissue. The lysates were cleared by centrifugation at $17,000 \times g$ for 5 min at 4°C. Five microliters (1 ng) of anti-c-Myc antibody (9E10; Santa Cruz Biotechnology) was added to 300 μ l of lysates, and the mixture was incubated at 4°C for 1 h. Then, 20 μ l of Dynabeads protein G (Life Technologies), equilibrated in the sampling buffer, was added to the samples, which were incubated at 4°C for 18 h. Finally, the beads were washed four times with 1 ml of sampling buffer to remove nonspecifically bound proteins. Specifically bound proteins were eluted by heating the beads with 1 $\times$ Laemmli sample buffer at 95°C for 5 min.

StrepIII-tag specific affinity purification. The purification was done as described in reference 9. Briefly, *N. benthamiana* leaves systemically infected with PVA^{NibSIII} and PVA^{VPgSIII} were harvested at 8 days postinfection (dpi) and homogenized at the ratio of 1 g of leaves per 2 ml of buffer (100 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 13% sucrose [pH 8.0]). A heavy membrane fraction was prepared from the homogenized leaf material by centrifugation. The membrane fraction was treated with 1% Triton X-100 and then subjected to Strep-tag specific affinity purification through Strep-tactin Sepharose (IBA). Bound proteins were eluted with buffer containing 2.5 mM desthiobiotin (100 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 2.5 mM desthiobiotin [pH 8.0]).

Agrobacterium-mediated delivery of constructs, Rluc-assay, and transient silencing of host genes. *Agrobacterium* isolates carrying different protein expression constructs and PVA icDNAs were infiltrated to the abaxial side of *N. benthamiana* leaves using a needleless syringe. The infiltration concentrations of *Agrobacterium* were as follows: OD₆₀₀ of 0.3 for silencing constructs of CK2, CPIP, CHIP, and HSP70; OD₆₀₀ of 0.5 for CP expression constructs during silencing and OD₆₀₀ of 0.1 when together with viral icDNA; OD₆₀₀ of 0.05 for PVA^{WT}::Rlucⁱ icDNA. The viral icDNA-carrying agrobacteria were infiltrated 1 day after the protein expression or silencing constructs. The control firefly luciferase construct (Fluc) was always infiltrated together with the Rluc-carrying viral icDNAs at OD₆₀₀ of 0.01. PVA gene expression was quantified by measuring *Renilla* luciferase (Rluc) activity 3 days after agroinfiltration of the virus. Rluc values were normalized against Fluc values using the following formula: normalized Rluc activity = (average Fluc activity/Fluc activity per sample) × Rluc activity per sample. Each experiment was done using at least four samples, and experiments were repeated at least twice. Error bars indicate standard deviations, and Student's *t* test was used to calculate the significance of differences between samples (*, *P* < 0.05; **, *P* < 0.01).

Western blotting. For the detection of CP mutant accumulation, the *Agrobacterium* isolates carrying CP^{WT}, CP^{AAA}, and CP^{ADA} expression constructs were infiltrated at an OD₆₀₀ of 1 and sampled 3 days postinfiltration. Standard Western blotting procedure was followed, and anti-CP antibody raised in rabbits was used for CP detection. For HSP70 and CK2 detection, a commercial polyclonal rabbit antibody against HSP70/HSC70 (9) and a polyclonal antibody raised in rabbits against *Nicotiana tabacum* CK2 α-subunit (19) were used.

Proteasome inhibition assay. *Agrobacteria* carrying PVA CP^{WT} expression construct were infiltrated into *N. benthamiana* leaves at an OD₆₀₀ of 0.3. To the same areas, a 50 μM solution of MG132 (Sigma-Aldrich) in water or water as a control was infiltrated 3 days later. After 24 h, samples were collected from the infiltrated leaves and a DAS-ELISA was performed to quantify PVA CP.

Quantitative RT-PCR. Total RNA was extracted from 100 mg of plant tissue using the GeneJET Plant RNA Purification minikit (Thermo Scientific). One microgram of extracted RNA was treated with 1 unit of RQ1 RNase-free DNase (Promega) at 37°C for 30 min. A reverse transcription reaction was performed on the DNase-treated RNA using the RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific) according to the manufacturers' recommendations. Five units of RNase H (New England BioLabs) was used to digest the remaining RNA in the cDNA at 37°C for 20 min. RNase H-treated cDNA was used in quantitative PCR using the DyNamo Flash SYBR green qPCR mastermix (Thermo Scientific) and the CFX96 Touch Real-Time PCR detection system (Bio-Rad). To verify silencing of CHIP and CPIP by qPCR primers for CHIP (5' GAG TGG GAG CAT GAA TCT AC 3' and 5' CAG GAA CCT CAG TTG GAG T 3') and CPIP (5' CAC AGA TGT TGT TAA GCC 3' and 5' TAG TCA ACA GTC CTG CC 3') were used. Protein phosphatase 2A (PP2A) gene with PP2A Forward primer (5' GAC CCT GAT GTT GAT GTT CG 3') and PP2A Reverse primer (5' GAG GGA TTT GAA GAG AGA TTT C 3') and F-box protein gene with F-box Forward primer (5' GGC ACT CAC AAA CGT CTA TTT C 3') and F-box Reverse primer (5' TGG GAG GCA TCC TGC TTA T 3') were used as internal controls (44).

Infection focus amount and size determination. PVA-GFP was infiltrated at an OD₆₀₀ of 0.0005 to initiate infection in widely spaced individual cells. Silencing and control constructs were infiltrated 1 day before at an OD₆₀₀ of 0.3. The infection initiation efficiency was assessed 3 dpi from virus infiltration by counting the visible infection foci reported by GFP fluorescence from digital micrographs of the infected plant leaves with the approximate area of 10.5 mm². The infection foci were measured from the same images. At least 10 areas were used. Images were recorded using an Axioskop 2 plus microscope (Carl Zeiss) with an AxioCam HRC microscope camera and AxioVision 4.8 software (Carl Zeiss). Student's *t* test was used to calculate the significance values, which are indicated as follows in the figures: *, *P* < 0.05; **, *P* < 0.01; no asterisk indicates that there is no statistical significance.

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