Analysis of Rubella Virus Capsid Protein-Mediated Enhancement of Replicon Replication and Mutant Rescue

Wen-Pin Tzeng, Jason D. Matthews, and Teryl K. Frey*

Department of Biology, Georgia State University, Atlanta, Georgia

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The rubella virus capsid protein (C) has been shown to complement a lethal deletion (termed ΔNotI) in P150 replicase protein. To investigate this phenomenon, we generated two lines of Vero cells that stably expressed either C (C-Vero cells) or C lacking the eight N-terminal residues (Δ8-Vero cells), a construct previously shown to be unable to complement ΔNotI. In C-Vero cells but not Vero or Δ8-Vero cells, replication of a wild-type (wt) replicon expressing the green fluorescent protein (GFP) reporter gene (RUBrep/GFP) was enhanced, and replication of a replicon with ΔNotI (RUBrep/GFP-ΔNotI) was rescued. Surprisingly, replicons with deleterious mutations in the 5′ and 3′ cis-acting elements were also rescued in C-Vero cells. Interestingly, the Δ8 construct localized to the nucleus while the C construct localized in the cytoplasm, explaining the lack of enhancement and rescue in Δ8-Vero cells since rubella virus replication occurs in the cytoplasm. Enhancement and rescue in C-Vero cells were at a basic step in the replication cycle, resulting in a substantial increase in the accumulation of replicon-specific RNAs. There was no difference in translation of the non-structural proteins in C-Vero and Vero cells transfected with the wt and mutant replicons, demonstrating that enhancement and rescue were not due to an increase in the efficiency of translation of the transcribed replicon transcripts. In replicon-transfected C-Vero cells, C and the P150 replicase protein associated by coimmunoprecipitation, suggesting that C might play a role in RNA replication, which could explain the enhancement and rescue phenomena. A unifying model that accounts for enhancement of wt replicon replication and rescue of diverse mutations by the rubella virus C protein is proposed.

Rubella virus (RUB), the causative agent of rubella and congenital rubella syndrome, is the sole member of the genus Rubivirus in the family Togaviridae of animal viruses (for a review, see reference 11). The rubella virus genome is a single-stranded, plus-polarity RNA of 9,762 nucleotides (nts) in length that contains two open reading frames (ORFs). The 5′-proximal ORF, the nonstructural protein ORF (NS-ORF), encodes two nonstructural proteins involved in virus RNA replication, P150 and P90 (the gene order is 5′-P150-P90-3′ within the ORF), while the 3′-proximal ORF, the structural protein ORF (SP-ORF), encodes the three virion proteins, the capsid protein (C) and envelope glycoproteins E1 and E2 (5′-C-E2-E1-3′ within the ORF). The NS-ORF is translated from the genomic RNA, while the SP-ORF, translated from a subgenomic (SG) RNA consisting of roughly the 3′ third of the genomic RNA. Both of these RNA species are transcribed from a genome-length RNA of minus polarity in infected cells.

The rubella virus C protein is a multifunctional protein. C is involved in several intermolecular interactions. First, it contains a motif between residues 28 and 56 (C is 300 amino acids [aa] in length) that binds the genomic RNA (16). Recent characterization has revealed that C is phosphorylated and that phosphorylation/dephosphorylation is important in genomic RNA binding and in encapsidation during virion formation (15). C is also able to modulate genomic and subgenomic RNA synthesis (31). Secondly, the C protein forms a disulfide-bonded homodimer in virions (2) and interacts with the cytoplasmic tails of E1 and E2 (12, 20, 34, 35). The 23 C-terminal aa of C, which serve as a signal sequence for E2, appear to mediate the interaction of C and the E2 and E1 glycoproteins at the site of budding in the Golgi (14). Thirdly, C has been shown to interact with two mitochondrial proteins, p32 and Par-4; the p32-binding domain within the C protein has been mapped to the N-terminal region of the protein (3, 18). While the function of these interactions in RUB replication has not been elucidated, p32 belongs to the family of cellular defense collagens (27). A recent report showed that C-p32 interactions were important both in mitochondrial redistribution in rubella virus-infected cells and in virus RNA synthesis (4). Finally, C induces apoptosis in RK13 cells, a cell line exquisitely sensitive to RUB-induced cytopathic effects, suggesting that it may play a role in the pathogenesis of RUB infection (9).

We recently discovered that C could complement a 500-nt, in-frame deletion between two NotI sites in the P150 gene and therefore termed ΔNotI (30). This effect was at an early time point in the replication cycle, before the accumulation of detectable virus-specific RNA (30). It was also reported that C could rescue rubella virus mutants in the 3′ cis-acting elements (CAE) and increase the replication efficiency of poorly replicating vaccine strains (8). In this study, we generated Vero cell lines that stably express the rubella virus C protein, characterized the rescue phenomenon in more detail, and proposed an underlying mechanism.

MATERIALS AND METHODS

Plasmids and recombinant DNA. We performed recombinant DNA manipulations essentially as described by Sambrook et al. (26) with minor modifications. Escherichia coli JM109 and DH5α were used as the bacterial hosts. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, Massachusetts). Restriction enzyme digestion and pDNA ligations were carried out essentially as described by Sambrook et al. (26). Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen, Valencia, Calif.). DNA sequencing was performed by the DNA Sequencing Laboratory at the Georgia State University.

* Corresponding author. Mailing address: Department of Biology, Georgia State University, 24 Peachtree Center Ave., Atlanta, GA 30303. Phone: (404) 651-3105. Fax: (404) 651-3105. E-mail: tfrey@gsu.edu.
MA) or Roche Molecular Biochemicals (Indianapolis, IN) and used as recommended by the manufacturers.

The following constructs were described previously: the RUB infectious cDNA clone Robo502 (29) and the replicons RUBrep/GFP, RUBrep/C-GFP, RUBrep/CAT-GFP, RUBrep/CAT-ΔNotI, RUBrep/CAT, and RUBrep/CAT-ΔNotI (28, 30). To make a mutation in RUBrep/GFP by replacement of the catalytic GIDD motif in the RNA-dependent RNA polymerase with AAA (RUBrep/GFP-GIDD*), we employed a three-round asymmetric PCR amplification, followed by a three-fragment ligation (29). In the first round, the mutagenic oligonucleotide 1340 (5′-GGCGGGATTTTCCAGGCGGCTATGTATCCTTCTC-3′; mutations are underlined) and the sequence is located at nts 5921 to 5995 of the genome) was used to prime asymmetric amplification on a PstI-linearized Robo502 template. In the second round, asymmetric amplification on the first-round PCR product as a template was primed with oligonucleotide 149 (5′-GGTCATGACATCATGACGCTCG-3′; the sequence is complementary to nts 6097 to 6131 of the genome, a region downstream from the PstI site at nt 6038 of the genome). In the third round, the second-round PCR product and oligonucleotide 106 (5′-AGGCTCAGCCGGCTAC-3′; the sequence is located at nts 5321 to 5340 of the genome, a region upstream from the BglII site at nts 5355 of the genome) were used to prime PCR amplification on a PstI-linearized Robo502 template. The BglII-PstI-digested PCR amplification product was ligated with the PstI-XbaI fragment from RUBrep/C-GFP and the BglII-XbaI fragment of RUBrep/GFP. To make a RUBrep/GFP construct containing a hemagglutinin (HA) epitope tag at the upstream NotI site in the P150 gene, RUBrep/HAGFP, we amplified by PCR a fragment using upstream oligonucleotide 1256 (5′-CCGGACACGGCCCCACCCGGCGGC-3′; the sequence is located at nts 788 to 811 of the genome, a region upstream from the PstI site at nt 816 of the genome) and downstream oligonucleotide 1340 (5′-GGCGGGATTTTCCAGGCGGCTATGTATCCTTCTC-3′; mutations are underlined) and HA sequence encoded; the sequence is complementary to nts 1661 to 1684 and 2192 to 2215 of the genome, and the HA sequence is between nts 1684 and 2192 of the genome) on a BglII-linearized Robo502 template. The amplification product was restricted with PinAI and NotI and ligated with PinAI-NotI-restricted RUBrep/GFP to create RUBrep/HAGFP-ΔNotI. The NotI-NcoI fragment of RUBrep/HAGFP-ΔNotI was then amplified into RUBrep/HAGFP-ΔNotI to generate RUBrep/HAGFP. To create a series of RUBrep/GFP constructs containing mutations in the 5′ untranslated region (UTR), we used the HindIII-PinAI fragment from Robo302 constructs containing these mutations (25) to replace the HindIII-PinAI fragment in RUBrep/GFP. To create a series of RUBrep/GFP constructs containing mutations in the 3′ UTR and 3′ CAEs, we used the Stul-EcoRI fragment from RUBrep/PAC constructs containing these mutations (8) to replace the Stul-EcoRI fragment in RUBrep/GFP. To produce RUBrep/GFP constructs with combinations of 5′ UTR and 3′ CAEs, we used the HindIII-PinAI fragment from the 5′ UTR RUBrep/GFP mutants to replace the HindIII-PinAI fragment in the 3′ CAE RUBrep/GFP mutants.

To generate the N-terminal FLAG epitope-tagged C protein constructs, C and CAΔS, both of which terminate at residue 277 of the C protein, we amplified a fragment by PCR using oligonucleotide 1243 (5′-TAAGGATATCCATGGACTATAAGGCACGACGACAGGATCTAACCTCCATCCATGC-3′; EcoRV site [underlined], initiating ATG, two FLAG sequences [emboldened], and nts 6539 to 6566 of the genome) as upstream primers and oligonucleotide 1012 (5′-GTACTCTAGATCCGGTGCTGGCCCAAGGATGG; XbaI site [underlined] and stop codon [underlined] followed, by a sequence complementary to nts 7324 to 7342 of the genome) as a downstream primer with an EcoRI-linearized Robo502 template. The amplification product was restricted with EcoRV and XbaI and cloned into pcDNA3.1 (+) (Invitrogen, Carlsbad, CA).

Establishment of clonally selected cell lines and immunofluorescence. The Vero cell lines stably expressing C and CAΔS were established by transfection with pcDNA3.1-C and pcDNA3.1-CAΔS plasmid DNA, followed by geneticin (Invitrogen) selection at a concentration of 1.2 mg/ml. Single-cell colonies were isolated and expanded. C protein production in clonally selected cell lines was assayed by Western blotting using anti-FLAG M2 monoclonal antibody (Sigma) as a probe. To analyze C construct localization in clonally selected cell lines, 1× 10⁶ Vero, C-Vero, or CAΔS-Vero cells were seeded onto coverslips in 35-mm culture dishes. Forty-eight hours after cells were seeded, the medium was removed, washed with phosphate-buffered saline (PBS), prefixed with PBS/ethanol (1:4) for 2 min, and then fixed with anhydrous ethanol for 5 min, followed by dehydration in acetone and air drying. As a control, we similarly fixed Vero cells infected with Robo502 rubella virus (multiplicity of infection, 5) at 48 h postinfection. The fixed cells were rehydrated with a graded PBS/ethanol series and then permeabilized with 0.1% saponin in PBS for 30 min, followed by blocking with 1% fetal bovine serum (FBS) in PBS for 30 min. Mouse anti-C monoclonal antibody (Viral Antigens, Inc.) was diluted 1:250 in 1% FBS/PBS and applied to slides for 1 h. A biotinylated secondary antibody (goat anti-mouse immunoglobulin G; Sigma) was diluted 1:2,500 in 1% FBS/PBS and applied to samples for 15 min. Streptavidin-conjugated Texas Red (Pierce) at a concentration of 14 mg/ml was then applied to each slide and allowed to incubate for 20 min. During the last 2 minutes of incubation with Texas Red solution, 1 μl of 10 mg/ml Hoechst 33342 (Molecular Probes) was added. Each coverslip was then washed seven to eight times with PBS, dehydrated with one ethanol wash followed by one acetone wash, and then air dried. After being dried, slides were mounted and analyzed using a Zeiss microscope with epifluorescence capacity and photographed with a Zeiss AxioCam.

In vitro transcription, transfection, and detection of viral RNA species. All plasmids were purified on CsCl isopycnic density gradients prior to their use. RUBrep constructs were linearized with EcoRI prior to in vitro transcription, which was carried out as previously described (29). The in vitro transcription reaction mixtures were used directly for transfection without DNase treatment or phenol–chloroform extraction. Cells were transfected with Lipofectamine 2000 (Invitrogen) as previously described (29). Total cell RNA was extracted from replicon-transfected cells 2 days posttransfection, and replicon-specific RNA species present were detected by Northern blotting using a NorthernMax-Gly kit (Ambion, Houston, TX) and nick-translated, 32P-labeled pGEM-GFP as a probe (28).

Immunoprecipitation, Western blotting, and coimmunoprecipitation. The protocols for immunoprecipitation (24) and Western blotting (33) have been described previously. For immunoprecipitation, Vero and C-Vero cells transfected with RUBrep/HAGFP in vitro transcripts were lysed at 3 days posttransfection. After a brief microcentrifugation, the supernatant was incubated with either anti-HA monoclonal antibody (Sigma, St. Louis, MO) or anti-FLAG M2 monoclonal antibody (Sigma). Subsequently, protein A-agarose beads (Roche Molecular Biochemicals) were added. Following 1 h of incubation, the agarose beads were washed three times, and bead-protein-antibody complexes were boiled for 5 min in Laemmli sample buffer to elute bound proteins. The immunoprecipitated proteins were resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8% SDS-PAGE), and the gel contents were electroblotted onto nitrocellulose membranes, followed by probing with anti-HA or anti-FLAG antibody.

RESULTS

Generation of stable cell lines. In our previous report (30), using replicons, we demonstrated that the rubella virus C protein could complement an in-frame, 500-nt deletion between two NotI sites in the P150 nonstructural gene. Complementation was observed only when the C protein was expressed from the SG RNA synthesized by the replicon containing the NotI deletion and when the C protein was expressed from a cotransfected replicon or from cotransfected plasmid. Deletion analysis showed that the 88 N-terminal amino acids of the C protein were necessary for complementation from a cotransfected plasmid. To further extend our analysis, we generated lines of Vero cells that stably expressed either residues 1 to 277 of the C protein, lacking the 23 C-terminal amino acids that serve as the signal sequence for E2, or residues 9 to 277 of the C protein, deleting the 8 N-terminal amino acids prior to the second in-frame AUG at the 5′ end of the C gene, a motif necessary for complementation (Fig. 1, top). Each construct was tagged with a double FLAG epitope at its N terminus. At least 30 clones selected G418-resistant cell lines expressing each construct were isolated and screened by Western blotting for expression of the modified C protein. Cell lines with robust but equivalent expression were selected to test for complementation of replication of transfected RUBrep/CAT-ΔNotI transcripts. As shown in Fig. 1 (bottom), the Vero cell line express-
ing residues 1 to 277 of the C protein (C-Vero) complemented replicon replication as detected by chloramphenicol acetyltransferase (CAT) expression, while the Vero cell line expressing residues 9 to 277 of the C protein (CΔ8-Vero) did not.

The rubella virus C protein enhances replicon replication, complements the NotI deletion, and rescues replicons with mutations in the 5′ UTR and 3′ CAE. While CAT expression as an indication of replicon replication is quantifiable, using green fluorescent protein (GFP) as a reporter gene, we could observe transfected cells daily for GFP expression without sacrificing the transfected cells. Shown in Fig. 2 is GFP expression in cultures of Vero, C-Vero, and CΔ8-Vero cells transfected with RUBrep/GFP, RUBrep/GFP-ΔNotI, and RUBrep/GFP-GDD*, a mutant in which the essential GDD triplet in the P90 RNA-dependent RNA polymerase was changed to AAA. GFP expression by RUBrep/GFP-ΔNotI was detected in C-Vero cells by 2 days posttransfection and was stronger by 4 days posttransfection but could not be detected in either Vero or CΔ8-Vero cells, confirming the complementation specific to C-Vero cells observed with RUBrep/CAT-ΔNotI. GFP expression by RUBrep/GFP-GDD* was not detectable in any of the three cell lines. GFP expression by RUBrep/GFP was detectable at 2 days posttransfection in all three cell lines but, surprisingly, was much stronger in C-Vero cells than in Vero or CΔ8-Vero cells. By 4 days posttransfection, GFP expression levels by RUBrep/GFP were equal in these three cell lines. Thus, the C protein also enhances replication of the wild-type (wt) replicon at early times posttransfection.

Using replicons expressing an antibiotic resistance gene to test rescue of 3′ CAE mutants, Chen and Icenogle (8) reported an increase in recovery of colonies following transfection and drug selection in cells expressing the RUB structural proteins in comparison to that of control cells. To confirm this finding, we constructed four mutant RUBrep/GFP replicons, each with one of the four stem loops in the 3′ CAE deleted. As recorded in Table 1, GFP expression by three of these four mutants was detectable by day 2 posttransfection in C-Vero cells and by all four mutants by day 4 posttransfection. In contrast, none of the mutant replicons expressed GFP in Vero or CΔ8-Vero cells at 2 days posttransfection, and GFP expression was observed only in these two cell lines transfected with the ΔSL1 and ΔS2 mutants by 4 days posttransfection. These results were consistent with our previous studies on viruses and replicons bearing these mutations (6, 7) in which ΔSL1 replicons exhibited a 100-fold drop in replication efficiency, ΔSL2 replicons exhibited a 3-fold drop in replication efficiency, and ΔSL3 and ΔSL4 replicons and viruses were not viable.

The 5′ UTR of the RUB genome also contains cis-acting elements which have different functions than do the 3′ CAE (e.g., it has been shown that mutations in the 5′ UTR reduce translation of the NS-ORF while mutations in the 3′ CAE do not [7, 25], and it is thought that 3′ CAE direct minus-strand RNA synthesis while cis-acting elements at the 5′ end of the genome are important in plus-strand RNA synthesis [11]). Therefore, to determine whether C protein rescue is specific for 3′ CAE mutants or also occurs with mutants at the 5′ end of the genome, we introduced a series of mutations into the 5′ UTR of RUBrep/GFP. As tabulated in Table 1, four of these five mutant replicons expressed GFP in C-Vero cells by 2 days posttransfection and all five expressed GFP by 4 days posttransfection. In contrast, only two of the five mutants expressed GFP in Vero or CΔ8-Vero cells by 4 days posttransfection. As with the 3′ CAE mutants, results for Vero cells were consistent with those that we previously reported when the same mutations were made in the virus (Table 1) (25). Thus, the C protein can rescue mutants in the cis-acting elements at either end of the RUB genome. To expand on this finding to see whether the C protein can rescue replicons with mutations in the cis-acting elements at both ends of the genome, we constructed replicons that combined one of the 5′ UTR mutations that were lethal in the virus D5 with each of the 3′ CAE SL deletion mutations. Replicons with three of the four 5′ UTR-3′ CAE double mutations were found to express GFP in C-Vero cells but not Vero or CΔ8-Vero cells by 4 days posttransfection. The nonviable combination mutant, D5 and ΔSL3, contained the most severe of the 3′ CAE mutations.

In our previous study (30), complementation of reporter gene expression by RUBrep/ΔNotI replicons by the C protein
correlated with accumulation of virus RNA demonstrable by Northern blotting, and RUBrep/GFP-ΔNotI genomic and SG RNAs were detectable in C-Vero cells but not in Vero or CΔ8-Vero cells (data not shown). Expression of GFP by the 3' CAE, 5' UTR, and 5' UTR-plus-3' CAE mutant replicons in C-Vero cells also correlated with production of detectable replicon RNA, and the level of GFP expression varied proportionately with the amount of replicon RNA detected (shown in FIG. 2. GFP expression and RNA synthesis by wt and mutant RUBrep/GFP in Vero cells and Vero cell lines expressing C protein. (A) Vero cells, C-Vero cells, and CΔ8-Vero cells were transfected with RUBrep/GFP, RUBrep/GFP-ΔNotI, or RUBrep/GFP-GDD* transcripts and monitored daily for GFP expression. Micrographs taken on days 2 and 4 posttransfection are shown. (B) Vero cells, C-Vero cells, and CΔ8-Vero cells were transfected with transcripts from RUBrep/GFP constructs with the following 3' CAE mutations: ΔSL1, ΔSL2, ΔSL3, or ΔSL4. At 2 days posttransfection, total cell RNA was isolated and assayed by Northern blotting for the accumulation of replicon-specific RNA species (G, genomic RNA). (C) C-Vero and Vero cells were transfected with RUBrep/GFP transcripts, and total cell RNA was isolated at 1, 2, 3, and 4 days posttransfection and assayed for the accumulation of replicon-specific RNAs.
FIG. 3. Subcellular localization of C protein in Vero cell lines stably expressing C protein constructs. Vero (A and E), C-Vero (B and F), CΔ8-Vero cells (C and G), and Vero cells infected with Robo502 virus (multiplicity of infection, 5 PFU/cell; fixed 48 h postinfection) (D and H) were costained with an anti-C (αC) monoclonal antibody (micrographs made using the same exposure) (A to D) and Hoechst 33342 (micrographs made using the same exposure) (E to H).

TABLE 1. Replication of wt and mutant replicons in C-expressing Vero cell lines

<table>
<thead>
<tr>
<th>Construct</th>
<th>Mutation/deletion</th>
<th>Virus phenotype</th>
<th>Result fora:</th>
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<tr>
<td></td>
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<td>Vero cells</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>GFP (2)</td>
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<tr>
<td>wt</td>
<td>GDD*</td>
<td>wt</td>
<td>+/−</td>
</tr>
<tr>
<td>GDD to AAA</td>
<td>NotI deletion</td>
<td>Lethal</td>
<td>-</td>
</tr>
<tr>
<td>3’ ΔS L1</td>
<td>9572–9647</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>3’ ΔS L2</td>
<td>9671–9702</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>3’ ΔS L3</td>
<td>9703–9730</td>
<td>Lethal</td>
<td>-</td>
</tr>
<tr>
<td>3’ ΔS L4</td>
<td>9742–9753</td>
<td>Lethal</td>
<td>-</td>
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<tr>
<td>5’ D 5</td>
<td>1–5</td>
<td>Lethal</td>
<td>+/−</td>
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<tr>
<td>5’ D 3</td>
<td>1–3</td>
<td>Lethal</td>
<td>-</td>
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<tr>
<td>5’ D 7</td>
<td>3–5</td>
<td>Down 2 logs</td>
<td>-</td>
</tr>
<tr>
<td>5’ D 6</td>
<td>2–3</td>
<td>Lethal</td>
<td>-</td>
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<tr>
<td>D5 + ΔS L1</td>
<td>ND</td>
<td>Lethal</td>
<td>-</td>
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<tr>
<td>D5 + ΔS L2</td>
<td>ND</td>
<td>Lethal</td>
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<tr>
<td>D5 + ΔS L3</td>
<td>ND</td>
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<td>D5 + ΔS L4</td>
<td>ND</td>
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aN Mutations from previous studies were introduced into RUBrep/GFP, and the ability of the mutant replicons to replicate was assayed in Vero, C-Vero, and CΔ8-Vero cells. The GDD* mutation was a substitution of the catalytic GDD triad in the RNA-dependent RNA polymerase (nts 5938 to 5946), the NotI deletion was in the P150 gene (28), the 3’ deletions were in the 3’ CAE (6, 7), the 5’ deletions were in the 5’ UTR (25), and the combination mutants contained the D5 5’ UTR deletion plus one of the 3’ deletions. The substitutions or nucleotides of the genome deleted in each mutation are given as are the phenotypes of each mutation in the infectious cDNA clone. The lethality of GDD mutations in the infectious clone was demonstrated by Wang and Gilliam (32). ND, not determined.

bCells transfected with each replicon construct were observed for GFP expression on days 2 and 4 posttransfection. RNA was extracted at 2 days posttransfection and assayed for the presence of replicon-specific RNAs by Northern blotting. Days posttransfection for GFP and RNA are indicated in parentheses. +, clearly detectable (GFP expression and RNA accumulation in C-Vero cells transfected with RUBrep/GFP at 2 days posttransfection in Fig. 2A and B); +/−, barely detectable (GFP expression and RNA accumulation in Vero and CΔ8-Vero cells transfected with RUBrep/GFP at 2 days posttransfection in Fig. 2A and B); −, not detectable.

Fig. 2B for the 3’ CAE mutants and summarized in Table 1 for all of the replicons). As shown in Fig. 2C, accumulation of wt RUBrep/GFP RNA was more rapid in C-Vero than in Vero cells. The amount of replicon RNA at 1 day posttransfection in RUBrep/GFP in C-Vero cells was roughly equivalent to the amount of replicon RNA at 3 days posttransfection in Vero cells, correlating with the earlier expression of GFP in C-Vero cells than in Vero cells. Thus, the enhancement of wt replicon replication and rescue of ΔNotI, 5’ UTR, and 3’ CAE mutant replicons in C-Vero cells are at a step in the replication cycle that affects accumulation of replicon-specific RNAs.

Mechanism of rescue. (i) Localization of expressed C and CΔ8 proteins. We next began analysis of the mechanism of C-mediated enhancement and rescue. Unexpectedly, when we used immunofluorescence to check the subcellular localization of the C protein in C-Vero and CΔ8-Vero cells (Fig. 3), we found that the CΔ8 protein localized in the nucleus (Fig. 3C) while the protein in C-Vero cells localized in the cytoplasm.
(Fig. 3B), as did C in rubella virus-infected Vero cells (Fig. 3D). Interestingly, the nuclei of CΔ8-Vero cells were smaller and were counterstained in a more intense and uniform fashion than were nuclei of Vero, C-Vero, or rubella virus-infected Vero cells. Since rubella virus and rubella replicon replication occur in the cytoplasm, the nuclear localization of the CΔ8 construct explains the lack of enhancement and rescue of wt and mutant replicons in CΔ8-Vero cells, and accordingly, these cells were not used in subsequent analyses.

**(ii) Efficiency of replicon transcript translation.** A common mechanism by which the C protein could enhance and rescue replication of wt, ΔNotI, and 5‘ UTR-3’ CAE mutant replicons is by increasing their translational efficiency. To test this hypothesis, we analyzed the production of the nonstructural proteins at an early time point following transfection to measure translational efficiency of the input transcripts before differences in translation due to different rates of replication occurred. For these experiments, a replicon variant was created in which an HA epitope was inserted in the upstream NotI site of the P150 gene (Fig. 1, top), allowing for detection of P150 by Western blotting using anti-HA antibody as a probe (in both the virus and replicons, the presence of this epitope had no effect on replication efficiency) (our unpublished observations). As shown in Fig. 4A, at 6 h posttransfection, similar levels of P150 production were detectable in Vero and C-Vero cells transfected with transcripts from wt-RUBrep/GFP and RUBrep/GFP containing the GDD* mutation and representative 5‘ UTR (D3) and 3‘ CAE (ΔSL1) mutations. P150 production was somewhat higher in RUBrep/GFP-ΔNotI-transfected cells, and the P150 band was stronger in C-Vero cells than in Vero cells. GFP was not detectable in any of the transfected cells at 6 h posttransfection, confirming that detectable replicon replication had not commenced at this time point. Overall, levels of P150 production from input transcripts were relatively equivalent among these replicons in the two cell lines and did not correlate with enhancement of replicon replication or mutant rescue. Thus, the C protein had no effect on translational efficiency of any of these replicon transcripts.

At 24 h posttransfection, GFP accumulation was clearly detectable in C-Vero cells transfected with RUBrep/GFP, RUBrep/GFP-D3, and RUBrep/GFP-ΔNotI, indicating replicon replication (Fig. 4B). However, the P150 band in RUBrep/GFP- and RUBrep/GFP-D3-transfected C-Vero cells was only slightly stronger than the P150 band in Vero cells transfected with these replicons or in both Vero cells and C-Vero cells transfected with either RUBrep/GFP-ΔSL1, which exhibits barely detectable GFP synthesis in C-Vero cells at this time point, or RUBrep/GFP-GDD*, which does not replicate. Thus, by 24 h posttransfection, most if not all of the P150 accumulation in cells transfected with these transcripts was due to translation...
from the input transcripts, even in cells in which replicon replication occurred. Interestingly, the greater accumulation of P150 in RUBrep/GFP-ΔNotI-transfected cells than in cells transfected with other replicons continued at 24 h posttransfection, and the relative difference was more pronounced. Since this enhancement of accumulation was observed in both Vero and C-Vero cells, it appears to be due to an enhanced translational efficiency or transcript stability specific to the NotI deletion.

(iii) Interaction of C protein with replicase components. Since the C protein did not have an effect on translational efficiency of replicon transcript-transfected cells, we next hypothesized that it played a role in the subsequent step in replicon replication, RNA replication (which could include generation of RNA replication complexes as well as initiation of RNA synthesis). Kujala et al. (13) previously showed that C protein and P150 colocalized in RUB-infected cells. We therefore tested for a P150-C protein interaction in C-Vero cells by coimmunoprecipitation. To this end, lysates from mock-transfected or RUBrep-HA/GFP-transfected C-Vero cells were immunoprecipitated with anti-HA (recognizing the HA-tagged P150) or anti-FLAG (recognizing the FLAG-tagged C protein) antibody, blotted, and then probed with each antibody. As shown in Fig. 5, both control reactions were successful: HA-tagged P150 was immunoprecipitated by anti-HA antibody in RUBrep-HA/GFP-transfected cells, while FLAG-tagged C protein was immunoprecipitated with anti-FLAG antibody. Additionally, in RUBrep-HA/GFP-transfected C-Vero cells, HA-tagged P150 was coimmunoprecipitated by anti-FLAG antibody and FLAG-tagged C protein was coimmunoprecipitated by anti-HA antibody, demonstrating an association between the C protein and P150.

**DISCUSSION**

The goal of this study was to investigate in more detail the ability of the RUB C protein to rescue RUB replicons with a lethal deletion in the P150 replicase gene. We initiated this study with the development of Vero cell lines stably expressing two forms of the C protein, extending from residues 1 to 277 (C-Vero cells) or 9 to 277 (CΔ8-Vero), both of which were FLAG epitope-tagged at the N terminus; both of these constructs lacked the 23 C-terminal amino acids of the C protein which serve as a signal sequence for the E2 protein. In accordance with our previous results, C-Vero cells could rescue the NotI deletion in the P150 gene while CΔ8-Vero cells could not; we subsequently found that the lack of rescue in CΔ8-Vero cells was due to localization of the CΔ8 protein in the nucleus. We also found that the replication of the wt replicon, RUBrep, was enhanced in C-Vero cells at early times (2 days) after transfection.

Chen and Icenogle (8) confirmed rescue of RUBrep/ΔNotI replicons in cell lines stably expressing the RUB SP-ORF and then made the unexpected observation that replicons with mutations in the 3′ CAE were also rescued. In this study, we found rescue of RUBrep/GFP mutants carrying a variety of 3′ CAE deletions in C-Vero cells and extended the observation to RUBrep/GFP mutants with deletions in the 5′ UTR and combination RUBrep/GFP constructs with both 5′ UTR and 3′ CAE deletions. Thus, the RUB C protein is able to enhance wt replicon replication and rescue mutant replicons with a striking diversity of deletions, namely, in the P150 gene and the 5′ and 3′ control elements. In all of these cases, C-mediated enhancement or rescue is at a basic step in replication that facilitates the accumulation of replicon RNAs. It should be noted, however, that whereas presence of the C protein gene in the subgenomic RNA of a replicon results in rescue, as evidenced by the finding that a ΔNotI virus replicates to titers near those of the wt virus, rescue of 5′ UTR and 3′ CAE mutants occurs only when the C gene is present on another template, such as a plasmid, and when viruses with 5′ UTR and 3′ CAE mutations exhibit impaired viability despite containing a C gene (6, 25).

We next used the C-stably expressing Vero cell lines to begin investigating the mechanism of C-mediated rescue. While the C protein in C-Vero cells was distributed in the cytoplasm, the CΔ8 protein was localized in the nucleus. Since RUB replication occurs in the cytoplasm, localization of CΔ8 to the nucleus would prevent it from acting directly on any step in the replication cycle, explaining its inability to rescue the replication of mutant replicons. We recently discovered that the C protein contains a stretch of four arginines (residues 60 to 63) that act as a nuclear localization signal when only the amino-terminal half (residues 1 to 151) of the protein is expressed (21). That the CΔ8 protein (residues 9 to 277) also localizes to the nucleus indicates that the eight N-terminal amino acids and residues in the C-terminal half of the protein are necessary to override the ability of stretches of basic amino acids in the C protein sequence, which are putatively present to interact with electronegative RNA in the capsid, to serve as nuclear localization signals. It should be noted that residues 277 to 300 of the C protein, which were lacking in the constructs used in this study, serve as the E2 signal sequence and mediate association of the full-length C protein with membranes and are also of importance in targeting the C protein in its native context (14).

The first mechanism of C-mediated rescue considered was that the C protein bound to replicon transcripts, resulting in an increase in transcript stability or enhancement of translation of the transcripts to produce the P150 and P90 replicase proteins. Based on a previous report (16), the C protein used in this study contains a domain between residues 28 and 56 that specifically binds to an RNA sequence in the P150 gene that is...
maintained in all of the replicon constructs. There is precedence in the literature for a virus capsid protein binding to enhance translation of a virus RNA, albeit a nonpolyadenylated RNA, with alfalfa mosaic virus (19). However, we found that translation of P150 from wt, GDDΔ, 5′ UTR (D3), and 3′ CAE (ΔSL1) mutant replicons in transfected Vero and C-Vero cells did not differ and that although translation of P150 from ΔNotI replicons was unexpectedly higher than from these other replicons, this was independent of the presence of the C protein. Thus, the C protein did not enhance translational efficiency of either wt or mutant replicon transcripts, and therefore, translational efficiency was not the basis of C-mediated rescue. In a previous study, we found that the ΔSL1 3′ CAE mutant had no effect on translation of the NS-ORF in vitro (7), consistent with our findings in this study. In another previous study using virus derived from infectious cDNA clones, we showed that several 5′ UTR mutations affected translation of the NS-ORF up to 10-fold (25). However, the D3 mutation used in this study was lethal and thus was not tested in the earlier study. Although transcript stability was not directly measured, it seems likely that differences in stability would be reflected in translational efficiency, and thus, we conclude that C-mediated rescue was also not due to transcript stabilization by the C protein.

The next step in the replication cycle at which C-mediated rescue could occur was RNA synthesis, which could include formation of the replication complex as well as initiation of RNA synthesis and removal of newly synthesized RNAs from the template. It was previously shown by immunofluorescence that C and P150 colocalized (13), and thus, we hypothesized that C-mediated rescue involved direct interaction of C with the viral replicase. This hypothesis was tested by coimmunoprecipitation, and it was found that in RUBrep/GFP-transfected C-Vero cells, P150 coimmunoprecipitated with C and, reciprocally, C coimmunoprecipitated with P150, consistent with the hypothesis. P90 also coimmunoprecipitated with P150 (10), and thus, C could interact with P150, with P90, or potentially with a cell factor associated with the replication complex. However, this is the first demonstration of a capsid protein of a plus-strand RNA virus interacting with a replicase protein. Whether this interaction is the key to the C-mediated rescue phenomenon will require mapping of the binding sites and the use of site-directed mutagenesis to determine whether abrogation of C-P150 association results in disappearance of the rescue effect.

Our working model that accounts for the strikingly diverse aspects of C-mediated rescue and enhancement of replicon replication is as follows. We hypothesize that the C protein and the NotI domain of P150 share a redundant function in the initial phases of viral RNA replication. C has now been shown to bind to both the viral RNA and the replicase proteins, and in doing so, it could both complement a deletion of the NotI domain and recruit replicon RNAs, including those with mutations in the cis-acting control elements, to the replication complex. That replicons with such mutations can replicate in the presence of C implies that these control elements are not necessary for RNA replication. While wt replicon replication can proceed without the presence of C, C is more efficient in its role in the initial phases of RNA replication than is the NotI domain, explaining the enhancement of wt replicon replication. In the context of virus infection, since rubella virus enters the cell by receptor-mediated endocytosis (23), following fusion of the virion membrane with an endosomal membrane, C protein in the capsid could remain bound to the virion RNA and subsequently recruit the replicase protein that is translated from the virion RNA by directly binding to it. This would localize the initial replication complex to the endosomal membrane, the site of RNA synthesis in rubella virus-infected cells (17). Subsequently, as infection proceeds and newly synthesized genomic RNAs migrate away from the initial replication complex and are translated, not necessarily accompanied by C, the resulting replicase proteins are able to initiate formation of replication complexes mediated by the NotI domain.

As a final note, coexpression of the nucleoprotein of coronaviruses either is necessary for viability or significantly enhances the infectivity of infectious cDNA clones and replicons (1, 5, 36, 37). Additionally, it was recently reported that the VP2 and VP3 structural proteins of the birnavirus, an infectious bursal disease virus, improved efficiency of replication of infectious cDNA constructs and recovery of the non-cell-culture-adapted virus (22). Thus, the participation of capsid proteins in virus replication may be a relatively general phenomenon among plus-strand RNA viruses.

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