SUPPLEMENTAL DATA

Picornavirus genome replication: Roles of precursor proteins and rate-limiting steps in oriI-dependent VPg uridylylation*

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Running Title: Picornavirus VPg uridylylation mechanism

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EXPERIMENTAL PROCEDURES

Construction of expression plasmids for 3BC, 3BCD, 3AB, and 3C^{Protease} – To make the expression plasmid for 3BC, oligos 1 – 4 (Table S1 lists all oligonucleotides used in this study; oligonucleotides were from Invitrogen life technologies and Integrated DNA Technologies, Inc.) were used to perform overlap-extension PCR using the viral cDNA (pMoRA, also known as pXpA-rib$^+$polyAlong (1)) as template. The 3BC region was cloned into the pET26Ub-C-His plasmid (2) using SacII and BamHI sites to give the pET26Ub-3BC-C147G-Chis plasmid. In order to change the tyrosine at the third amino acid position in 3BC to phenylalanine, oligos 2 and 5 were used to amplify the 3BC region from the pET26Ub-3BC-C147G-Chis plasmid. This fragment was cloned into the pET26Ub-Chis plasmid using SacII and BamHI sites to obtain the pET26Ub-3BC-Y3F-C147G-Chis. To make the 3BCD expression plasmid, oligos 1, 17, 3, and 4, were used to perform overlap-extension PCR using the viral cDNA (pMoRA) as template. The PCR product was disgested and cloned into the pET26Ub-3CD-C147G-Chis plasmid (Arnold and Cameron, unpublished results) using the SacII and AflII sites to give the pET26Ub-3BCD-C147G-Chis plasmid. In order to clone 3AB, oligos 10 and 11 were used for PCR to amplify the 3AB region with pMoRA as template. The PCR product was cloned into pET26Ub using SacII and EcoRI sites to obtain pET26Ub-3AB.

In order to generate an active 3C protease clone, oligos 8 and 9 were used to amplify the 3C region using pMoRA as template. The PCR product was cloned into the pET26Ub-N-His plasmid using the BamHI and EcoRI sites. The pET26Ub-N-His plasmid is designed such that an N-terminal 6-histidine tag is produced when cloned into the BamHI site. The resulting plasmid was labeled pET26Ub-3C^{Protease}-Nhis. DNA sequencing at the Penn State Nucleic Acid Facility was used to verify the integrity of the above clones.

Expression and purification of 3BC, 3BC-Y3F, 3BCD, 3CPro, 3AB, 3Dpol and, 3C – 3Dpol and 3C were purified as described previously (2). Wild-type 3BC and the 3BC Y3F mutant were expressed using the ubiquitin fusion system described previously (3). BL21(DE3)pCG1 cells containing either the pET26Ub-3BC-C147G-Chis or the pET26Ub-3BC-Y3F-C147G-Chis plasmid were grown overnight at 30 ºC in 100 mL of NZCYM supplemented with kanamycin at 25 μg/mL (K25), chloramphenicol at 20 μg/mL (C20), and dextrose at 0.1%. The overnight cultures were used to seed 2 L of NZCYM K25/C20-supplemented media to an OD_{600} of 0.05. Cells were grown at 37 ºC until an OD_{600} of 1 was reached. Cells were chilled to 25 ºC and induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 500 μM. Cells were grown for 4 h at 25 ºC and harvested. Cell pellets were weighed and stored at –80 ºC. Frozen cell pellets were thawed on ice and suspended in lysis buffer 1 (100 mM potassium phosphate, pH 8.0, 20% glycerol, 10 mM 2-mercaptoethanol (β-ME), 5.6 μg/mL pepstatin A, 4 μg/mL leupeptin, 0.5 mM EDTA, and 60 μM $ZnCl_2$) at a concentration 4 mL of buffer per g of cell pellet. Cells were homogenized using a Dounce homogenizer; cells were lysed by passing through a French pressure cell at a pressure per square inch of 20,000. Phenylmethyl-sulfonyl fluoride (PMSF) and nonidet P-40 (NP-40) were added after lysis to final concentrations of 2 mM and 0.1% v/v, respectively. Polyethylenimine (PEI) was slowly added to a final concentration of 0.25% v/v to the lysate in order to precipitate nucleic acid. The lysate was stirred slowly at 4 °C for 30 min and then centrifuged in a Beckman JA-30.50 Ti rotor for 30 min at 24,000 rpm at 4 °C. The PEI supernatant was diluted 5-fold, conductivity adjusted to 50 mM NaCl, and loaded at 1 mL/min onto a phosphocellulose (Whatman, P-11) column that was equilibrated with buffer A (50 mM HEPES, pH 7.5, 20% glycerol, 10 mM β-ME, and 0.1% NP-40) containing 50 mM NaCl. Approximately 1 mL of resin bed volume was used per 20 mg of total protein. Protein concentration was measured by using the BioRad protein assay. The column was washed to baseline with buffer A containing 50 mM NaCl and protein

was eluted by using a six-column-volume, linear gradient from 50 to 2 M NaCl in buffer A. Fractions (1/10th the bed volume of the column) were collected, assayed for purity by SDS-PAGE, and pooled. The protein concentration of the pool was adjusted to 1 mg/mL; the pool was loaded onto a Ni-NTA Agarose (Qiagen) column equilibrated with buffer A containing 1 M NaCl and 20 mM imidazole. Approximately 0.5 mL resin bed volume was used per 50 mg total protein. The column was washed with 20 column volumes of buffer A containing 1 M NaCl and 20 mM imidazole. Protein was eluted from the Ni-NTA column using buffer A containing 1 M NaCl and 500 mM imidazole. Fractions (0.5 mL) were collected until the concentration of the eluted protein fell below the desired value. The high NaCl and imidazole concentrations were reduced from the fractions by dialysis against buffer A containing 200 mM NaCl using Spectra/Por dialysis tubing with a MWCO of 6000 – 8000 Da. SDS-PAGE was used to assess the purity of the eluted fractions. Protein concentration was determined by using the following extinction coefficients: 0.008960 $\mu M^{-1} \cdot cm^{-1}$ (3BC-C147G) and 0.007680 $\mu M^{-1} \cdot cm^{-1}$ (3BC-Y3F-C147G). These values were determined by using the protein parameters tool on the ExPASy site http://us.expasy.org/tools/protparam.html). The absorbance values were measured at 280 nm in 6 M guanidine-HCl, pH 6.5. Conductivities of the fractions were measured; fractions were then aliquoted, and stored at – 80 °C.

3BCD was expressed as described above for 3BC. Frozen cells pellets were and lysed in lysis buffer 1 containing 500mM NaCl. PMSF and NP-40 were added after lysis to final concentrations of 2 mM and 0.1% v/v, respectively. PEI was as added as described above for the 3BC purification. After centrifugation, the PEI supernatant was saved and ammonium sulfate was added to 40% saturation and centrifuged again for 30 min at 24,000 rpm at 4 °C. The ammonium sulfate pellet was resuspended in 40 mL ammonium sulfate resuspension buffer (50 mM HEPES pH 7.5, 20% glycerol, and 500 mM NaCl). Protein concentration was determined by using the BioRad protein assay and the concentration was adjusted to 1mg/mL prior to loading onto a 1 mL Ni-NTA agarose column pre-equilibrated with 10 column volumes of Ni equilibration buffer (same as ammonium sulfate resuspension buffer). Bound protein was eluted with Ni elution buffer (50 mM HEPES pH7.5, 20% glycerol, 500 mM NaCl, and 500 mM imidazole). SDS-PAGE was used to assess the purity of the eluted fractions. The protein concentration of fractions was determined by measuring the absorbance values at 280 nm in 6 M guanidine-HCl, pH 6.5 using an extinction coefficient of 0.008469 $\mu M^{-1} \cdot cm^{-1}$. Conductivities of the fractions were measured; fractions were then aliquoted, and stored at – 80 °C.

The 3CPro was expressed as described above for 3BC, however, only a 100 mL culture was used. Cell pellets were thawed and lysed as described above using lysis buffer 1 lacking EDTA and $ZnCl_2$. PMSF and NP-40 were added after lysis to final concentrations of 2 mM and 0.1% v/v, respectively. The lysates were clarified by centrifugation in a Beckman JA-30.50 Ti rotor for 30 min at 24,000 rpm at 4 °C. The lysate supernatants were passed over Ni-NTA Spin columns (Qiagen). The columns were equilibrated prior to loading and washed after loading with buffer A containing 500 mM NaCl according to the manufacture's protocol. Protein was eluted in two 100-µL fractions using buffer A containing 500 mM NaCl and 500 mM imidazole. Protein concentrations were determined by measuring the absorbance at a wavelength of 280 nm using the same extinction coefficients as used for the wild-type proteins. Conductivities of the fractions were measured and the fractions were then aliquoted and stored at – 80 °C.

3AB was also expressed using the ubiquitin fusion system. BL21(DE3)pCG1 cells containing the pET26Ub-3AB plasmid were grown overnight at 30 ºC in 100 mL of NZCYM supplemented with K25, C20, and 0.1% dextrose. The overnight cultures were used to seed 1 L of NZCYM K25/C20/0.1% dextrose-supplemented media to an OD_{600} of 0.05. Cells were grown at 37 ºC until an OD_{600} of 1.5 was reached. Cells were chilled to 25 ºC and induced by addition of IPTG to a final concentration of 500 µM. Cells were grown for 4 h at 25 ºC and harvested. Cell pellets were weighed and stored at – 80 ºC. 3AB purification was performed using a modified version of a protocol described previously (4). Frozen cell pellets were thawed and lysed as described above using lysis buffer 2 (50 mM Tris-

HCl, pH 7.6, 20% glycerol, 1 mM DTT, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 0.5 mM EDTA, and 100 mM NaCl). PMSF was added after lysis to a final concentration of 1 mM. The lysate was centrifuged in a Beckman Ti-60 rotor for 30 min at 30,000 rpm at 4 °C. The pellet was suspended in 10 mL of solubilization buffer (50 mM Tris-HCl, pH 8.5, 20% glycerol, 1 mM DTT, 1 mM EDTA, 50 mM NaCl, 0.5% NP-40) and centrifuged in a Beckman Ti-60 rotor for 30 min at 30,000 rpm at 4 °C. The supernatant was saved and this extraction procedure was repeated two more times. The second and third supernatants containing 3AB were combined and the conductivity and protein concentration was adjusted to 25 mM and 1 mg/mL, respectively, using dilution buffer (Tris-HCl, pH 8.5, 20% glycerol, 1 mM DTT, and 0.1% NP-40). This diluted pool was then passed through a 10 mL DEAE-cellulose (Whatman, DE52) column connected in tandem to a 5 mL S-sepharose (GE Healthcare) column. DEAE was cycled following the manufacture's protocol prior to use. Both columns were equilibrated with the dilution buffer containing 25 mM NaCl. 1 mL of DEAE resin was used for every 10 mg of total protein and 1 mL of S-sepharose was used for every 20 mg of total protein in the initial pool. The S column was then washed using buffer S (25 mM MOPS, pH 7.2, 20% glycerol, 1 mM β-ME, 0.1% NP-40) containing 50 mM NaCl. Fractions were eluted using buffer S containing 200 mM NaCl. Protein concentrations were determined as described above using an extinction coefficient of 0.012090 $\mu M^{-1} \cdot cm^{-1}$. Conductivities of the fractions were measured; fractions were then aliquoted, and stored at – 80 °C.

Transcription and purification of 61nt oriI – OriI for the VPg uridylylation reaction was transcribed from pUC18-61nt-oriI plasmid (2) linearized using the *Bst*Z17 I site. A 4-ml transcription reaction (40 mM HEPES, pH 7.5, 32 mM magnesium acetate, 40 mM DTT, 2 mM Spermidine, 28 mM NTPs, 25 μg/mL linearized template, and 25 μg/mL T7 RNA polymerase) at 37 ºC for 2 h was followed by removal of magnesium pyrophosphate by centrifugation for 2 minutes. The supernatant was treated with RQ1DNase (1 U/μg of template; Promega) for 30 min to remove the template; two phenol/chloroform extractions followed by a chloroform extraction were performed to deproteinate the RNA. Next, the RNA was precipitated with ammonium acetate, washed with 70% ethanol, and the pellet was suspended in 50% formamide. This provided the starting material for gel purification. The entire volume was loaded onto a 10% acrylamide, 50% formamide gel (18 cm × 24 cm × 2 mm). The gel was run at 25 mA for approximately 4 h (the band corresponding to the full length oriI had migrated to the middle of the gel by this time, as determined by the migration of bromophenol blue and xylene cyanol indicator dyes). The oriI band was excised from the gel by using UV-shadowing using a TLC plate with a fluorescent indicator (PEI Cellulose F, EM Science). The gel piece was cut into tiny squares and placed in an Elutrap electrophoresis chamber (Schleicher & Schuell). The eluted RNA was precipitated with ammonium acetate, washed with 70% ethanol, and suspended in Tris-EDTA. This was then passed over two Sephadex G-25 (Sigma) spin columns. RNA concentration was calculated by measuring absorbance at 260 nm. The extinction coefficient was calculated for the 61-nt oriI plus three guanosine residues that are transcribed at the 5' end of oriI (0.749400 $\mu M^{-1} \cdot cm^{-1}$) (5).

OriI for the RNA-filter binding assay was transcribed as above, however, only a 40 μL reaction was performed and $[\alpha\text{-}^{32}P]$UTP (0.33 μM final concentration) was included in the reaction. Following DNase treatment, the RNA was spun through a Micropure-EZ centrifugal filter (Amicon Bioseparations, Millipore) to remove any protein from the RNA according to the manufacturer's instructions. The RNA was precipitated with ammonium acetate; the pellet was suspended in Tris-EDTA and then passed through two G25 Sephadex spin columns to remove free nucleotides. To confirm RNA quality and sufficient removal of nucleotides, analysis by PAGE and a Typhoon 8600 scanner (Molecular Dynamics) in the storage phosphor mode was performed.

Construction of mutated replicons clones – Mutation of the Gln-Gly cleavage site between 3B and 3C to Gly-Gly in the poliovirus replicon, pRLucRA (1,6), required overlap-extension PCR amplification using oligos 6, 7, 12, and 13 and the pRLucRA plasmid as template. The amplified

fragment was cloned into the pRLucRA plasmid using the SpeI and BglII sites. Screening for the introduction of a new NheI site identified correct clones. The clone was labeled pRLucRA-3B-3C-Gly-Gly. The NheI site was introduced via a silent mutation in the forward oligo (oligo 12). In order to verify that this silent mutation did not produce any adverse effects, oligos 12 and 13 were used to amplify this region from the pRLucRA plasmid. The amplified fragment was cloned into the pRLucRA plasmid and screened for the new NheI site. This new replicon, referred to as pRLucRA-3B-3C-WT, was used as the wild-type replicon for all of the replicon studies comparing the Wildtype and the Gly-Gly mutant. The tyrosine at the third position of 3B was changed to phenylalanine by using overlap-extension PCR amplification with oligos 12 – 15 and pRLucRA-3B-3C-Gly-Gly as template. Correct clones were identified as before. The resulting clone was labeled pRLucRA-3B-3C-Y3F-Gly-Gly. DNA sequencing at the Penn State Nucleic Acid Facility using pRLuc-BglII-r oligo (oligo 13) as primer, was used to verify the presence of the 3B-3C processing site mutation and the Y3F mutation.

Transcription of subgenomic replicons and luciferase assays – RNA transcripts were generated from the pRLucRA plasmids after linearization with ApaI. Transcription reactions, typically 20 µL, consisted of 350 mM HEPES, pH 7.5, 32 mM magnesium acetate, 40 mM DTT, 2 mM Spermidine, 28 mM NTPs, 0.5 µg template, and 0.5 µg T7 RNA polymerase. Reactions were incubated at 37 ºC for 3 h followed by removal of magnesium pyrophosphate. DNase treatment with RQ1DNase (Promega) was used to remove the template; lithium chloride precipitation of the RNA was used to remove unincorporated nucleotides. RNA concentration was calculated by measuring absorbance at 260 nm, assuming an A_{260} of 1 was equivalent to 40 µg/mL.

Luciferase assays were performed as described previously (2) with slight modifications as described here. Only 5 µg of replicon RNA were used to electroporate 1.2×10^6 cells. Cells were suspended in normal growth media (DMEM/F12 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 5 mL/1 $\times 10^6$ cells) following electroporation. Light production was quantified for 10 seconds by using a Junior LB 9509 luminometer (Berthold Technologies). Protein concentration of the lysate supernatant was determined by using the BioRad Protein Assay reagent following the manufacture's instructions. Relative light units (RLU) per µg of protein is reported.

Western-blot analysis of replicon proteins – Cells were transfected with replicon RNA as described above. Following 20 h of incubation at 34 °C, cells (1×10^6) were pelleted by centrifugation at 14,000 × g for 2 min and lysed in 1× cell culture lysis reagent (CCLR, Promega, 50 µL). Luciferase activity and protein concentration were measured for the samples as described above. For Western blot analysis, an equal volume of 2× SDS-PAGE dye (225 mM Tris, pH 6.8, 5% SDS, 50% glycerol, 5% β-ME and 0.05% bromphenol blue) was added to the lysed cells. An equal amount of RLU/µg of protein was used for Western blot analysis. Proteins were separated by using 8% and 15% SDS-PAGE as indicated in the figure legend; transfer to nitrocellulose membrane (Osmonics, Inc. and/or GE Healthcare) was performed using the Genie transfer unit from Idea Scientific Company (Minneapolis, MN) for 50 min at 24 V in transfer buffer (25 mM Tris-Glycine/3 mM SDS/20% (v/v) methanol (pH 8.3)); dry milk (5% (w/v)) in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) was used to block the membrane (the blocking and probing steps were performed at room temperature for 1 h). Polyclonal anti-sera used to probe for viral proteins were produced in rabbit against purified recombinant poliovirus proteins 3D, 3C-His, and 3AB-His by Covance Research Products, Inc. (Denver, PA). Anti-sera were capable of detecting at least 100 pg of purified protein at 1:1,000 dilutions in TBS-T (1:1000 for the anti-rabbit HRP secondary). 3D (PA 473) anti-serum was used at a 1:10,000 dilution in TBS-T; 3C (PA 474) and and 3AB (PA 485) anti-sera were used at 1:5,000 dilutions in TBS-T. Immunocomplexes were detected by using a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) at a 1:10,000 dilution in TBS-T and the ECL system (GE Healthcare). Kodak Biomax MR film was used to visualize the chemiluminescence.

Generation and purification of polyclonal PV VPg, PV 3C and HCV NS5A antisera – N-terminally and C-terminally KLH-conjugated VPgs were used to raise polyclonal antisera in rabbits. The following VPg segment was used as antigen: TGLPNKKPNVPTIRTAKVQ. The two antisera (PA 797 and PA 796, respectively) were combined and purified as described below. Purified PV 3C-His, PV 3D and HCV NS5A-His were used to raise polyclonal antisera in rabbit (PA 474, PA 473, and PA 413, respectively). Antisera were precleared by centrifugation at 16000 × g at 4 °C for 15 min. Ammonium sulfate was then added to 33% saturation over a 30 min period while the antisera were stirred in the cold room. After addition of all of the ammonium sulfate, the mixture was stirred an additional 30 min. The precipitated material was pelleted by centrifugation at 50000×g at 4 °C for 30 min. The pellet was suspended in load buffer (20 mM Tris, pH 8.0, 50 mM NaCl) to approximately the same volume as the starting antisera volume and dialyzed overnight at 4 °C against the load buffer. Conductivity of the dialyzed sample was adjusted to 50 mM by dilution using the load buffer containing no NaCl. This sample was then passed through a column packed using DEAE Affi-gel Blue gel (BioRad). A bed volume of 1-mL resin per 5 mg of total protein was used. The column was prepared by washing with 5 column volumes of pre-wash buffer (100 mM acetic acid, 1.4 M NaCl, and 40% isoprpanol) and then 10 column volumes of the load buffer. Fractions (one-third the column volume) were collected. Fractions were analyzed by SDS-PAGE and protein concentration measured using the BioRad assay. Fractions containing IgGs were pooled and concentrated approximately 40-fold using a YM10 Amicon Centriprep centrifugal concentrator (Millipore). Protein concentration was measured using the BioRad assay; sodium azide was added to a final concentration of 0.1%, and the samples were aliquoted and placed at – 80 °C. The purified antisera were capable of detecting 100 pg of purified protein at a 1:1,000 dilution in TBS-T (1:1,000 for the anti-rabbit HRP secondary).

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Table S1. *Oligonucleotides used in this study*

Restriction sites are shown in bold; codons containing nucleotide changes are italicized.

No.	Name	Sequence
1	3B-SacII-f	5'-GCG GAA TTC **CCG CGG** TGG AGG AGC ATA CAC TGG TTT-3'
2	3C-Chis-BamHI-r	5'-GCG GGT ACC **GGA TCC** TTG ACT CTG AGT GAA GTA-3'
3	3C-C147G-f	5'-CCA ACC AGA GCA GGA CAG *GGT* GGT GGA GTC ATC ACA-3'
4	3C-C147G-r	5'-TGT GAT GAC TCC ACC *ACC* CTG TCC TGC TCT GGT TGG-3'
5	3B-Y3F-SacII-f	5'-GGC T**CC GCG G**TG GAG GAG CA*T TC*A CTG GTT TAC CAA AC-3'
6	3B-3C-QGtoGG-f	5'-CGG ACA GCA AAG GTA *GGA* GGA CCA GGG TTC GAT TAC-3'
7	3B-3C-QGtoGG-r	5'-GTA ATC GAA CCC TGG TCC *TCC* TAC CTT TGC TGT CCG-3'
8	3C-N-His-BamHI-f	5'-GCG **GGA TCC** TCC GGA GGA CCA GGG TTC GAT TAC GC-3'
9	3C-PmeI-EcoRI-r	5'-GCG **GAA TTC GTT TAA AC**T TAC TAT TGA CTC TGA GTG AAG TA-3'
10	3A-SacII-f	5'-GCG GAA TTC **CCG CGG** TGG AGG ACC ACT CCA GTA T-3'
11	3B-EcoRI-r	5'-GCG **GAA TTC GGA TCC** TTA CTA TTG TAC CTT TGC TGT CCG-3'
12[a]	pRLuC-SpeI-f	5'-GCG **ACT AGT** TAT TAT AAC TAG GAA CTA TGA AGA CAC CAC AAC AGT **GCT** ***AGC*** TAC CCT GGC CCT TC-3'
13	pRLuc-BglII-r	5'-CGC **AGA TCT** CCA CTT CTT TGC CA-3'
14	pRLucRA-3B-Y3F-f	5'-GGA CAC CAG GGA GCA *TTC* ACT GGT TTA CC-3'
15	pRLucRA-3B-Y3F-r	5'-GGT AAA CCA GT*G AA*T GCT CCC TGG TGT CC-3'
16	3D-AflII-f	5'-AAC GAT CCC AGG **CTT AAG** ACA GAC TTT GAG-3'
17	3D-AflII-r	5'-AAA GTC TGT **CTT AAG** CCT GGG ATC G-3'
18	3D-KpnI-r	5'-TTG AGT TAA AAA TTG A**GG TAC C**TG AGC AGC CAG AT-3'

[a] An NheI site was introduced via a silent mutation