

The 3A Protein from Multiple Picornaviruses Utilizes the Golgi Adaptor Protein ACBD3 To Recruit PI4KIII β

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The activity of phosphatidylinositol 4-kinase class III beta (PI4KIII β) has been shown to be required for the replication of multiple picornaviruses; however, it is unclear whether a physical association between PI4KIII β and the viral replication machinery exists and, if it does, whether association is necessary. We examined the ability of the 3A protein from 18 different picornaviruses to form a complex with PI4KIII β by affinity purification of Strep-Tagged transiently transfected constructs followed by mass spectrometry and Western blotting for putative interacting targets. We found that the 3A proteins of Aichi virus, bovine kobuvirus, poliovirus, coxsackievirus B3, and human rhinovirus 14 all copurify with PI4KIII β . Furthermore, we found that multiple picornavirus 3A proteins copurify with the Golgi adaptor protein acyl coenzyme A (acyl-CoA) binding domain protein 3 (ACBD3/GPC60), including those from Aichi virus, bovine kobuvirus, human rhinovirus 14, poliovirus, and coxsackievirus B2, B3, and B5. Affinity purification of ACBD3 confirmed interaction with multiple picornaviral 3A proteins and revealed the ability to bind PI4KIII β in the absence of 3A. Mass-spectrometric analysis of transiently expressed Aichi virus, bovine kobuvirus, and human klassevirus 3A proteins demonstrated that the N-terminal glycines of these 3A proteins are myristoylated. Alanine-scanning mutagenesis along the entire length of Aichi virus 3A followed by transient expression and affinity purification revealed that copurification of PI4KIII β could be eliminated by mutation of specific residues, with little or no effect on recruitment of ACBD3. One mutation at the N terminus, I5A, significantly reduced copurification of both ACBD3 and PI4KIII β . The dependence of Aichi virus replication on the activity of PI4KIII β was confirmed by both chemical and genetic inhibition. Knock-down of ACBD3 by small interfering RNA (siRNA) also prevented replication of both Aichi virus and poliovirus. Point mutations in 3A that eliminate PI4KIII β association sensitized Aichi virus to PIK93, suggesting that disruption of the 3A/ACBD3/PI4KIII β complex may represent a novel target for therapeutic intervention that would be complementary to the inhibition of the kinase activity itself.

Reorganization of cellular membranes has been recognized as a critical aspect of replication of positive-stranded RNA viruses (29). Positive-stranded RNA viruses use membranes from distinct cellular organelles to concentrate and protect RNA replication machinery from cellular defenses. Among the picornaviruses, poliovirus and other enteroviruses devote their 3A and 2BC genes to reorganizing cellular membranes associated with the Golgi apparatus (37). Consistent with reorganization of the Golgi, the 3A proteins from multiple enteroviruses are also responsible for the shutdown in cellular secretion associated with enteroviral infection (7). Recent work has suggested that the binding of the protein Golgi-specific brefeldin A resistance guanine nucleotide exchange factor 1 (GBF1) by enteroviral 3A is required for the secretion phenotype and viral replication (2, 21).

Recent work has also demonstrated the importance of the phosphatidylinositol 4-phosphate (PI4P) composition of membranes associated with positive-stranded-RNA replication (17). This model suggests that GBF1 binding of poliovirus 3A proteins is important vis-à-vis recruitment of phosphatidylinositol 4-kinase class III, catalytic subunit β (PI4KIII β), to replication of complex membranes. In this model, the change in phosphoinositol membrane lipid composition resulting from PI4 kinase activity is expected to directly recruit the viral-RNA-dependent RNA polymerase via its PI4P-binding domain. The requirement for PI4 kinase activity has also been demonstrated in enterovirus 71 (1). Furthermore, two known antienteroviral drugs have been shown to have anti-PI4K activity, supporting the notion that PI4KIII β inhibitors may have promise as broad-spectrum picornavirus

therapeutics. However, the family Picornaviridae is highly diverse, and the relationship between other members of this family and PI4KIII β is unclear.

Among the most divergent animal picornaviruses are those that belong to the kobuvirus genus, including Aichi virus (32, 38). Originally isolated in Japan from patients with oyster-associated gastroenteritis in 1991, Aichi virus has a worldwide reach with a relatively high seroprevalence (32, 38). Several other kobuviruses, including bovine, porcine, sheep, canine, murine, and chiropteran (bat) strains, have been recovered from stool in the past decade, as well as a kobuvirus-like agent of a closely related *Picornaviridae* genus, klassevirus/salivirus (12, 14, 18, 22, 23, 30, 31, 39).

In this study, we characterized associations between the picornavirus nonstructural protein 3A and host factors using a mass spectrometry-based proteomic approach. We found that transiently expressed Strep-Tagged 3A proteins from Aichi virus and bovine kobuvirus both copurified PI4KIII β and a Golgi adaptor

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protein, acyl-CoA binding domain protein 3 (ACBD3 or GCP60), under stringent capture and wash conditions. In the process, we found that Aichi virus 3A is myristoylated at its N-terminal glycine, despite the fact that it lacks a recognizable N-myristoyltransferase (NMT) motif. By Western blot detection, we found ACBD3 to be stably associated with several picornaviruses, including poliovirus, Aichi virus, bovine kobuvirus, porcine kobuvirus, human rhinovirus 14, and coxsackie B viruses. We also found that the association with PI4KIII β and 3A proteins was more stable with Aichi virus and bovine kobuvirus 3A proteins than with other picornaviral 3A proteins. Affinity purification of ACBD3 in the absence of 3A revealed association with PI4KIII β , and consistent with this, all picornaviral 3A proteins that could recruit PI4KIII β also copurified with ACBD3. By alanine-scanning mutagenesis of Aichi virus 3A, we subsequently identified residues within the protein necessary for interaction with PI4KIII β or ACBD3. We further demonstrated the necessity of PI4KIII β activity and association for Aichi virus replication through chemical and genetic means. Overall, our data suggest that multiple picornaviral 3A proteins utilize ACBD3 to recruit PI4KIII β .

METHODS

Virus, cells, plasmids, and cloning. The complete Aichi virus genome (8,280 nucleotides) was synthetically constructed (Bio Basic Inc., Markham, Ontario, Canada) and inserted into a plasmid-based expression construct (pAV-UCSF; GenBank accession no. [JQ281544](#)), based on pAV-FL (GenBank accession no. [AB040749](#)) (34), and is freely available upon request. 293T and HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM)-H21 medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. All genes for transient transfections were cloned into a modified pcDNA4/TO vector with a C-terminal 2 \times Strep-Tag (26) or a Flag tag, as noted in Results.

Cloning of viral 3A genes was performed using the InFusion Advantage kit (Clontech) and sequence confirmed using the BigDye system on an Applied Biosystems 3130xl sequencer. Mutagenesis of single or multiple sites in Aichi virus 3A was performed as described previously (9, 13). Primer sequences are provided in Table S1 in the supplemental material.

Affinity purifications. Affinity purifications were carried out as previously described (16). Briefly, 10 μ g of plasmid was transfected with FuGeneHD (Roche, Basel, Switzerland) into a 15-cm plate of log-phase 293T cells at 50% confluence. Cells were harvested 48 to 72 h later in 10 mM EDTA-phosphate-buffered saline (PBS) on ice, centrifuged for 5 min at 140 RCF (relative centrifugal force) in an Eppendorf 5410 centrifuge at 4°C, and lysed with 1 h of nutation at 4°C in 2 ml of capture buffer: 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA buffer containing 0.5% NP-40. DNA was pelleted for 15 min at maximum speed in an Eppendorf 5415D centrifuge at 4°C. Lysate was precleared by rotation for 1 h at 4°C with 50 μ l of protein G Sepharose 4 FastFlow (Amersham) and resuspended 1:1 in the capture buffer described above without added detergent. Preclarification beads were pelleted for 2 min at 2,000 rpm, and lysate was affinity purified by inversion mixing overnight at 4°C with 45 μ l of StrepTactin Sepharose resuspended 1:1 in detergent-free capture buffer. Beads were washed three times in capture buffer containing 0.1% NP-40, with a final wash in detergent-free capture buffer for a total wash time of 20 to 30 min. Captured proteins were eluted with 1 \times D-desthiobiotin (IBA Technology, Göttingen, Germany) dissolved in 40 μ l capture buffer by inversion mixing for 30 min at room temperature. For magnetic bead affinity purifications, no preclearing was performed, and 2 ml cell lysate was incubated with 40 μ l of StrepTactin magnetic bead slurry (Qiagen) for 2 h and then washed three times in capture buffer containing 0.1% NP-40, with a final wash in detergent-free capture buffer for a total wash time of approximately 5 to 8 min. Proteins were eluted as described above.

For Western blot analysis, 10 μ l of eluate was run on a 4 to 12% bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Invitrogen, Carlsbad, CA), transferred to a nitrocellulose membrane using the XCell Blot II system, and blotted with 1:300 PI4KIII β A-2 antibody (sc-166822; Santa Cruz), 1:250 ACBD3 518 antibody (sc-101277; Santa Cruz), 1:300 GBF1 25 antibody (sc-136240; Santa Cruz), 1:1,000 anti-Strep-Tag antibody (Qiagen), or 1:1,000 anti-Flag tag antibody (Qiagen). Blots were stained in 1:10,000 Alexa 680 anti-mouse IgG secondary antibody (Invitrogen), imaged on a Licor Odyssey imager, and quantitated using Licor Odyssey 2.1 software.

Protein identification by mass spectrometry. Protein identification from affinity-purified samples was performed using peptide sequencing by mass spectrometry. Affinity-purified samples containing approximately 10 to 20 μ g total protein were denatured with urea, reduced, alkylated with dithiothreitol (DTT) and iodoacetamide, and subjected to in-solution digestion using sequencing-grade porcine trypsin (Promega). For targeted posttranslational modification analysis, alternate digests were additionally performed using GluC or AspN (Roche). The resulting peptide samples were desalted using C₁₈ ZipTips (Millipore). Secondary validation of ACBD3 and PI4KIII β proteins in affinity-purified samples was obtained by in-gel digestion of selected protein bands in silver-stained SDS-PAGE gels using standard protocols (in-gel digestion protocol, University of California at San Francisco), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) peptide sequencing.

Two systems were used for LC-MS/MS analysis. The first was a linear trap quadrupole with Fourier transform ion cyclotron resonance (LTQ-FT) mass spectrometer (Thermo) equipped with a 10,000-lb/in² nano-Acquity ultraperformance liquid chromatography system (Waters) for reversed-phase chromatography with a C₁₈ column (BEH130; 1.7- μ m bead size, 100 μ m by 100 mm). The second system was a linear ion trap LTQ instrument (Thermo) equipped with an Ultimate high-performance liquid chromatograph and Famos autoinjector (LC Packings) and a self-packed C₁₈ column (New Objective Inc.; 5- μ m bead size, 100 μ m by 150 mm). The two LC systems were operated at flow rates of 600 and 300 nl/min, respectively, and peptides were separated using a linear gradient over 42 min from 2% to 30% B; solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. On the LTQ-FT instrument, survey scans were recorded over a 310 to 1,600 *m/z* range, and MS/MS was performed in data-dependent acquisition mode with CID fragmentation on the six most intense precursor ions, measured in the ion trap. On the LTQ instrument, survey scans were taken over 320 to 1,500 *m/z*, and the top three ions in the survey scan were subjected to a high-resolution MS scan of the precursor and then a CID (collision-induced dissociation) fragmentation MS/MS scan.

Mass spectrometry peak lists were generated using in-house software called PAVA, and data were searched using Protein Prospector software, v. 5.8.0 (5). Database searches were performed against the *Homo sapiens* plus *Picornaviridae* subset of the NCBI nonredundant (nr) Refseq database (date accessed, 14 January 2011), to which were added virus clone sequences missing from the public database, totaling 37,526 entries. This database was concatenated with a fully randomized set of 37,526 entries for estimation of the false-discovery rate (10). Data were searched with a parent mass tolerance of 20 ppm on the LTQ-FT or 0.8 Da on the LTQ and a fragment mass tolerances of 0.8 Da for both instruments.

For database searching, peptide sequences were matched as tryptic peptides with no missed cleavages and with carbamidomethylated cysteines as a fixed modification. Variable modifications included oxidation of methionine, N-terminal pyroglutamate from glutamine, loss of methionine, and N-terminal acetylation. For reporting of protein identifications from this database search, score thresholds were selected that resulted in a protein false-discovery rate of 1.1%. The specific Protein Prospector parameters were a minimum protein score of 22, a minimum peptide score of 15, and maximum expectation values of 0.02 for protein and 0.05 for peptide matches. Protein identification results from specific affinity purification experiments are reported with a spectral count as an approxima-

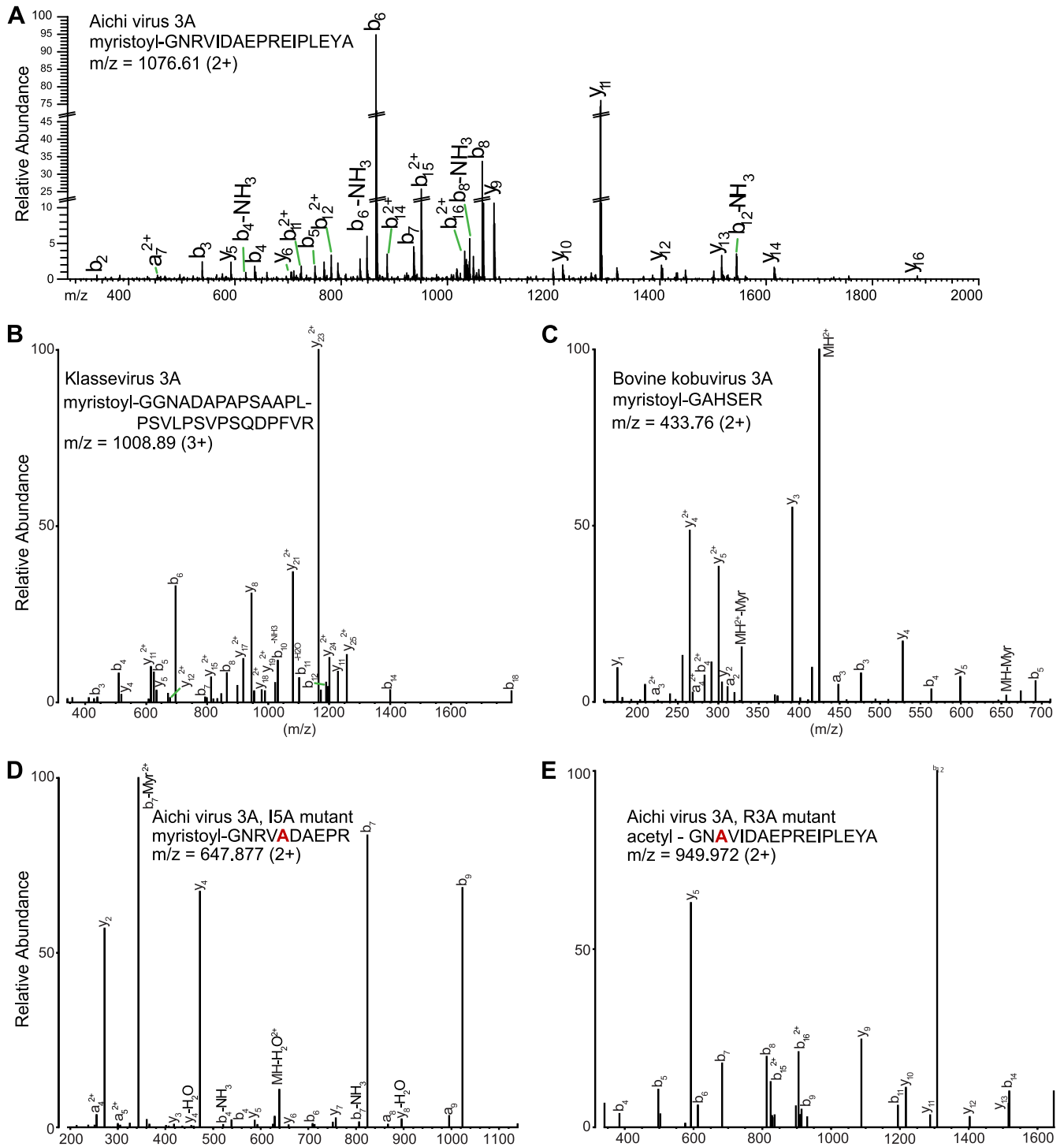


FIG 1 The N termini of Aichi virus 3A, klassevirus, and bovine kobuvirus 3A are myristoylated. Strep-Tagged kobuvirus 3A proteins were transiently transfected into 293T cells, affinity purified using the Strep-Tag system, and then subjected to mass-spectrometric peptide sequencing. Mass spectra from LC-MS/MS analysis show myristoylation of Aichi virus 3A (A), klassevirus 3A (B), bovine kobuvirus 3A (C), and I5A mutant Aichi virus 3A (D); however, only the acetylated form was observed for the Aichi virus 3A R3A mutant (E).

tion of protein abundance, along with percent sequence coverage and an expectation value for the probability of the protein identification (8, 24).

To address nonspecificity of background interacting proteins in the affinity purifications, multiple capture experiments were performed for 91 unrelated picornavirus protein constructs selected from 21 subspecies

and 11 different genes, totaling 293 control data sets. The control proteins included both structural (VP0, VP1, VP2, VP3, and VP4) and nonstructural (L, 2A, 2C, 2D, 3C, and 3D) genes from Aichi virus, poliovirus, theiloviruses, enterovirus, and klassevirus. These control experiments were used as a background model for defining interaction specificity of

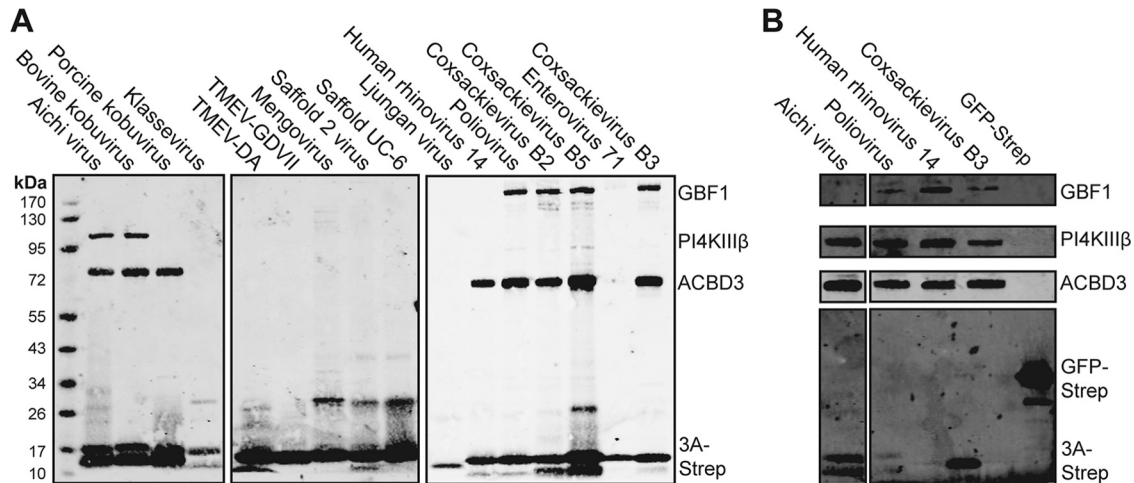


FIG 2 Picornaviral 3A proteins interact differentially with PI4KIII β and ACBD3. (A) Strep-Tagged 3A proteins across the picornavirus family were affinity captured under fully equilibrated binding conditions and then blotted with anti-ACBD3, anti-PI4KIII β , and anti-Strep-Tag antibodies, revealing stable interactions between kobuvirus and enterovirus 3A proteins and ACBD3. (B) When the affinity capture conditions were changed to rapid capture and wash conditions (described in Materials and Methods), transient interactions between the enterovirus 3A proteins and PI4KIII β were also observed.

copurifying proteins for a given prey 3A protein. Using peptide counts as an approximation of protein abundance, Z-scores were calculated for all copurified proteins to represent their interaction specificity to the bait. For each interacting protein in a replicate, a population of peptide counts consisting of the observed counts in the replicate together with the observed counts in all each of the control experiments was used to derive a per-replicate protein Z-score by calculating the number of standard deviations that the protein's peptide counts in the replicate were above or below the population mean. Per-replicate protein Z-scores were then averaged to obtain a final Z-score for each prey protein. Z-scores for proteins interacting with Aichi virus 3A were calculated using four replicate analyses of the viral protein affinity purification results together with a background model of 293 control, non-3A data sets.

For mapping of potential posttranslational modifications (PTMs) on the 3A bait proteins themselves, alternate digests using AspN or GluC instead of trypsin were analyzed using targeted analysis. In these searches, additional posttranslational modifications were allowed, including N-myristoylation, phosphorylation of serine, threonine, or tyrosine, and GlyGly as a signature of ubiquitinylation on Lys. Nonspecific (one) and missed cleavages (increased to two) were also allowed, as would be consistent with GluC and AspN reaction conditions. The peptide expectation value threshold was increased to 0.001, and spectrum matches were manually validated to eliminate false-positive identifications. Complete mapping of the Aichi virus 3A wild-type construct is reported in a peptide summary in Table S2 in the supplemental material. The most significant biological PTMs were found to be myristoylation and acetylation of the N-terminal glycine. All identified myristoylated peptides in kobuvirus 3A proteins were manually verified (sample spectra are provided in Fig. 1).

Viral luciferase replicon assay. *Renilla* luciferase replicons were created using the synthetic virus plasmid pAV-UCSF or XpA by replacing the capsid region of each virus with the *Renilla* luciferase gene using InFusion Advantage cloning (Clontech, Takara Bio). Primer sequences are described in Table S1 in the supplemental material. Two micrograms of plasmid was cleaved for 1 to 3 h at 37°C, using HindIII-HF for the Aichi virus replicon and MluI for the XpA replicon (New England BioLabs) and then purified over a Zymo DNA-5 column (Zymo Research). One microgram of cut plasmid was T7 amplified for 4 h, Turbo DNase cleaved for 15 min, and purified over a Zymo RNA-25 column (Zymo Research).

For replicon assays, 20,000 293T or HeLa cells were plated the night before in 100 μ l complete medium per well in an opaque, white 96-well plate (Grenier America; catalog no. 655075). Cells were transfected with

100 ng of T7-amplified RNA using a TransIT mRNA transfection kit (Mirus Bio) in 75 μ l complete medium mixed with 1 \times EnduRen live-cell imaging substrate (Promega). Cells were maintained in an incubator and analyzed for *Renilla* luciferase activity hourly on a Veritas microplate luminometer. After viral replication had peaked, the total cell count was determined by CellTiterGlo assay (Promega), and luminometer readings were normalized to the cell count.

shRNA knockdown. Previously published short hairpin RNA (shRNA) oligonucleotides specific to PI4KIII β and GFP were ligated and cloned into a modified pSicoR lentivirus packaging vector (4). The sequence-confirmed shRNA-expressing pSicoR plasmids were cotransfected with pRSV and pVSG plasmids into 293T cells, and lentivirus was harvested 72 h after transfection. Lentivirus was used to infect 293T cells, shRNA-expressing clones were selected for with 1 μ g/ml puromycin, and expression was confirmed by mCherry expression. Target gene knockdown was confirmed by Western blotting and quantitative reverse transcription-PCR (qRT-PCR). For qRT-PCR, 2 μ g of total RNA from 293T cells was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT) (19), and qRT-PCR was performed with 480 DNA SYBR green I Master Mix (Roche) on a LightCycler (Roche).

siRNA knockdown. Control, PI4KIII β , GBF1, and ACBD3 ON-TARGETplus small interfering RNAs (siRNAs) were purchased commercially (Dharmacon). In a 96-well plate, 3,000 log-phase HeLa cells were reverse wet transfected in 50 nM siRNA with 0.15 μ l of Dharmafect 1 transfection reagent and 125 μ l total medium per well. At the same time, 60,000 log-phase HeLa cells were reverse wet transfected in 50 nM siRNA with 3 μ l of Dharmafect 1 transfection reagent and 2.5 ml total medium per well in a six-well plate to assay for gene knockdown. After 72 h, cells in the 96-well plate were transfected with viral replicon RNA and measured for *Renilla* luciferase as described above. After the viral replicon assay was finished (~12 h), total cell count was normalized using CellTiterGlo to adjust for differences in growth among different siRNAs. All measurements are averages from six wells per viral RNA-siRNA pair and were adjusted to subtract background *Renilla* luciferase signal from the first time point (50 min) due to the higher *Renilla* luciferase background in HeLa cells.

RESULTS

Aichi virus, bovine kobuvirus, and klassevirus 3A proteins are myristoylated. To identify proteins that interact with the main

TABLE 1 Highest specificity picornavirus 3A-human protein-protein interactions, ranked by Z-score^a

Source of bait 3A	Prey protein		Mean z score	Replicate counts ^b
	Accession no.	Name or description		
Aichi virus	311771621	Phosphatidylinositol 4-kinase beta isoform 2	17.12	5 14 9 16
Aichi virus	15826852	Golgi resident protein GCP60	17.06	33 23 10 25
Aichi virus	4504341	Histone acetyltransferase type B catalytic subunit	12.84	7 0 3 1
Hrv14	15826852	Golgi resident protein GCP60	16.29	2 15 15 30
Hrv14	296317339	Voltage-dependent anion-selective channel protein 2 isoform 2	11.91	0 6 7 13
Poliovirus	313747582	Golgi-specific brefeldin A resistance guanine nucleotide exchange factor 1 isoform 2	17.12	30 19 15 14
Poliovirus	21361794	Cullin-associated NEDD8-dissociated protein 1	17.12	2 1 4 2
Poliovirus	15826852	Golgi resident protein GCP60	17.03	34 30 13 9
Klassevirus	82659109	E3 ubiquitin-protein ligase UBR4	16.55	6 15 3 12
Klassevirus	5453998	Importin-7	14.09	2 1 1 6
Klassevirus	16445419	Secretory carrier-associated membrane protein 3 isoform 1	13.16	6 2 1 9
Klassevirus	6912734	Transportin-3 isoform 1	12.84	3 2 0 7
Klassevirus	229577398	Basic leucine zipper and W2 domain-containing protein 2	12.84	2 1 0 6
Klassevirus	21361794	Cullin-associated NEDD8-dissociated protein 1	12.84	1 2 0 5
Porcine kobuvirus	94721252	Vesicle-associated membrane protein-associated protein A isoform 2	17.12	12 13 19 22
Porcine kobuvirus	4504341	Histone acetyltransferase type B catalytic subunit	17.12	3 1 7 6
Porcine kobuvirus	19923919	Receptor expression-enhancing protein 6	17.12	2 1 2 3
Porcine kobuvirus	82659109	E3 ubiquitin-protein ligase UBR4	17.11	67 44 97 94
Porcine kobuvirus	15826852	Golgi resident protein GCP60	17.11	42 46 39 46
Porcine kobuvirus	6005794	PRA1 family protein 2	14.72	2 2 3 3
Bovine kobuvirus	311771621	Phosphatidylinositol 4-kinase beta isoform 2	17.12	17 17 14 19
Bovine kobuvirus	19923919	Receptor expression-enhancing protein 6	17.12	5 6 5 7
Bovine kobuvirus	164519076	Transmembrane 9 superfamily member 4 precursor	17.12	4 5 7 12
Bovine kobuvirus	115430112	Receptor expression-enhancing protein 5	17.12	4 1 6 8
Bovine kobuvirus	82659109	E3 ubiquitin-protein ligase UBR4	17.12	88 72 110 171
Bovine kobuvirus	15826852	Golgi resident protein GCP60	17.11	49 52 51 57

^a Interacting proteins were identified using in-solution digestion of eluted proteins followed by mass spectrometry. Hits were weighted by the Z-score of the peptide counts of captured proteins from four biological replicate experiments against a control database of picornavirus protein affinity purifications that did not include tagged 3A proteins (see Materials and Methods). Based on the Z-score of the peptide count, PI4KIII β and ACBD3 were the top two interacting proteins with Aichi virus 3A.

^b Values are reported as spectral counts, as described in Materials and Methods, where vertical bars delineate values from independent experiments.

membrane-reorganizing protein of picornaviruses, protein 3A, we undertook an unbiased screen using single-step affinity purification of C-terminally Strep-Tagged 3A proteins in 293T cells followed by mass-spectrometric peptide sequencing analysis of in-solution trypsin digests. Careful inspection of the mass spectrometry data identified a myristoylation on the N-terminal glycine of Aichi virus 3A, bovine kobuvirus 3A, and klassevirus 3A (Fig. 1). The activity of N-myristoyltransferase (NMT) enzymes is strongly dependent on the first five residues, with an N-terminal glycine being absolutely required (27). The N-terminal sequence of Aichi virus 3A is GNRVIDAE. Although algorithms such as NMT—The Myr Predictor (<http://mendel.imp.ac.at/myristate/SUPLpredictor.htm>) and the ExPASy Myristoylator do not predict the N terminus of any of the kobuviruses to be NMT substrates, *in vitro* experiments using human NMT1 have shown synthetic octapeptides such as GNRAAARR to be valid substrates with kinetics comparable to those of other known substrates (3, 33).

To explore the functional effects of myristoylation, N-terminal mutations of the Aichi virus 3A protein were analyzed. In Aichi virus 3A, the G1A mutation abolishes myristoylation, as do N2A and R3A, while V4A, I5A, D6A, and E8A mutants retain N-terminal myristoylation, as measured by mass spectrometry analysis of the affinity-purified protein. Mass spectra for the I5A and R3A mutants of Aichi virus 3A are shown in Fig. 1D and E, and unique peptide identifications for this analysis are provided in Table S3 in the supplemental material. Targeted analysis of MS

spectra for affinity purifications of 3A proteins derived from coxsackieviruses B2, B3, and B5, enterovirus 71, poliovirus 1, and human rhinovirus 14 did not reveal evidence of N-terminal myristoylation, despite the fact that significant peptide counts for unmodified or acetylated N termini were recovered. The cardioviruses, theilovirus strains TMDA, BeAn, and TMGDVII, and Saf-fold viruses UC6 and Saf2 have serines as their start residues and thus cannot be substrates for NMTs. For all 3A affinity purification experiments, additional searches were performed for other posttranslational modifications, including phosphorylations, GlyGly signatures for ubiquitination, and broad-mass-range modifications of up to 500 atomic mass units. With the exception of N-terminal acetylation, cysteine carbamidomethylation, pyroglutamylation of glutamine, and oxidation, no other modifications were detected in these experiments.

The 3A protein was also frequently observed to run as a doublet at 15 and 17 kDa by SDS-PAGE (for an example, see Fig. 2A) with detection by silver staining or by anti-streptavidin tag antibody in Western blot format. In the case of Aichi virus 3A, mass-spectrometric analysis confirmed that both bands contained full-length 3A protein, the lower band comigrating with streptavidin. Despite extensive searches, no posttranslational modifications, including myristoylation, could be found that explain a mass shift in these bands, suggesting that these may be conformationally resolved forms of the protein. N-terminal and C-terminal peptides for wild-type and mutant Aichi virus 3A proteins are provided in Table S4 in the supplemental material.

Method for determining specific interactions from mass spectrometry data. To identify proteins that specifically interact with picornavirus 3A within the set of proteins identified by mass spectrometry, we first attempted to use a Strep-Tagged GFP as a negative control for nonspecific interactions. The combined protein identifications, across four replicate experiments for Aichi virus 3A and GFP, resulted in approximately 70 and 40 putative interacting proteins, respectively (see Tables S5 and S6 in the supplemental material). Based on these results, we determined that Strep-Tagged GFP did not adequately sample the spectrum of nonspecific interactions in this experimental system, consistent with observations by other groups (6, 16).

Therefore, we chose instead to perform a comprehensive analysis of background proteins, assessing their ability to interact with multiple unrelated viral proteins, consisting of 91 unique non-3A picornaviral bait proteins assayed in 293 individual experiments (see Materials and Methods). The set of background interacting proteins was then used to derive the specificity for virus bait-host protein interactions, ranked using Z-scores. To minimize false positives, we report interactions that pass a highly conservative Z-score threshold of 10 and whose preys are represented by a minimum of two peptides in at least two biological replicates. To further strengthen confidence in the analysis, Aichi virus 3A affinity purifications were compared against those of 3A proteins from 15 diverse picornaviruses. The most specific protein interaction partners for each 3A protein were ranked using the Z-score metric and resulted in a refined list of candidate interactions (Table 1). This method of scoring interactions was in part confirmed by the top ranking of GBF1 for poliovirus and coxsackievirus 3A proteins. Prior to this study, GBF1 was the only confirmed protein to copurify with any picornaviral 3A. The complete table of Z-scores for all identified interacting proteins across all picornaviral 3A proteins tested is reported in Table S7 in the supplemental material.

Affinity purification-MS (AP-MS) of C-terminally Strep-Tagged picornaviral 3A proteins copurifies PI4KIII β and ACBD3. The top-ranked protein identified in affinity purifications with both Aichi virus 3A and bovine kobuvirus 3A was PI4KIII β (Table 1). PI4KIII β interaction with Aichi virus 3A and bovine kobuvirus 3A was confirmed by Western blot (Fig. 2A). Intriguingly, one peptide to PI4KIII β was found in one replicate of coxsackievirus B5 affinity purification by MS, and a weak positive result by Western blot (Fig. 2A) was also observed. To investigate whether other 3A proteins such as poliovirus 3A might still interact with PI4KIII β more transiently, affinity purifications for selected enteroviral 3A proteins were repeated under more rapid kinetic conditions, using short binding and washing steps and capture on magnetic StrepTactin beads (6). Using this more rapid procedure, PI4KIII β was detected by affinity purification with 3A proteins from poliovirus, human rhinovirus 14, and coxsackievirus B3 (Fig. 2B). We note that the 3A protein from rhinovirus 14 also captured GBF1, consistent with HRV sensitivity to brefeldin A (15).

The second-ranking protein identified in the Aichi virus 3A affinity purifications was acyl-CoA binding domain protein 3 (ACBD3), also known as Golgi complex-associated protein GCP60 (Table 1). This protein was also affinity purified specifically by the 3A proteins of multiple picornaviruses, including poliovirus, Aichi virus, bovine kobuvirus, porcine kobuvirus, human rhinovirus 14, and coxsackie B viruses. Although the 3A

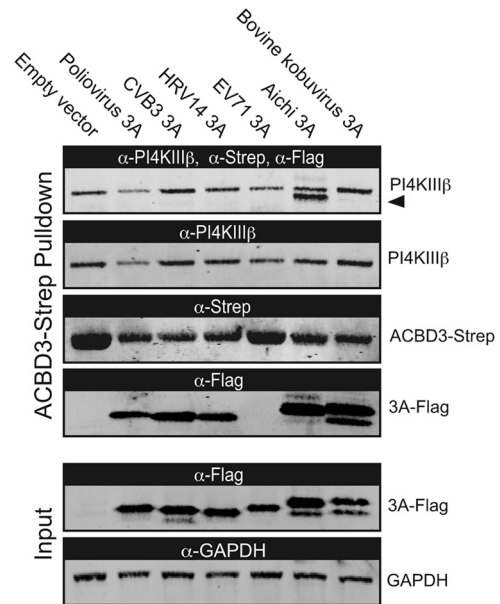


FIG 3 Affinity purification of Strep-Tagged ACBD3. Strep-Tagged ACBD3 and FLAG-tagged enterovirus and kobuvirus 3A proteins were transiently cotransfected into 293T cells, and ACBD3 complexes were affinity captured under rapid kinetic conditions. Samples were then serially Western blotted for PI4KIII β , Strep-Tag, and Flag tag. ACBD3 copurifies with PI4KIII β in the absence or presence of picornavirus 3A. In the presence of 3A, ACBD3 copurifies with 3A proteins from multiple entero- and kobuviruses, with the exception of EV71. In the presence of Aichi virus 3A, an SDS-resistant complex consistent with ACBD3 bound to 3A is visible (black triangle). A separate Western blot of the input samples was serially blotted for GAPDH and Flag-tag (bottom) to control for expression and loading levels.

protein of EV71 did not copurify with ACBD3 under these conditions, we note that it did copurify with a different acyl-CoA binding protein, ACAD9. Although it was not detectable by Western blotting (Fig. 2A), a single peptide for ACBD3 was detected in an EV71 3A protein AP-MS experiment using rapid capture and wash steps on StrepTactin Sepharose beads (data not shown); thus, we cannot exclude interaction of EV71 with ACBD3. ACBD3 is a Golgi resident protein that has been implicated in multiple cell signaling systems, including Golgi complex maintenance, steroidogenesis, and apoptosis (11). Interaction between ACBD3 and picornaviral 3A proteins was confirmed by Western blotting (Fig. 2A). Furthermore, Aichi virus 3A and PI4KIII β were both immunoprecipitated by anti-ACBD3 antibody in Aichi virus 3A-transfected 293T cells, as detected by mass spectrometry (see Table S8 in the supplemental material). However, in the absence of Aichi virus 3A, endogenous PI4KIII β and ACBD3 did not coprecipitate with each other, suggesting that 3A specifically stabilizes the complex containing both of these proteins (see Table S8).

To test whether ACBD3 and PI4KIII β have direct interactions in the absence of Aichi virus 3A, C-terminally Strep-Tagged ACBD3 was transiently transfected and affinity purified using the rapid binding and washing protocol with and without a series of 3A proteins from entero- and kobuviruses. In the absence of any transfected 3A proteins, PI4KIII β was found to copurify with ACBD3 (Fig. 3, empty vector lane), indicating that the interaction between ACBD3 and PI4KIII β does not require 3A. Affinity purification of ACBD3 in the presence of 3A proteins captured 3A from poliovirus, coxsackievirus B3, human rhinovirus 14, Aichi

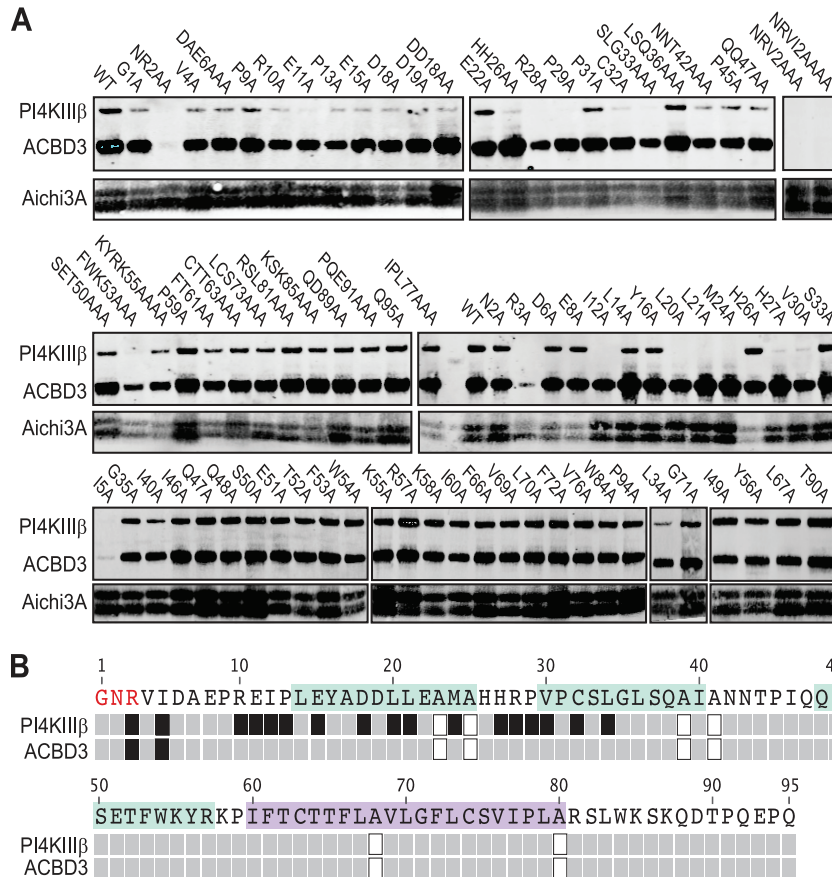


FIG 4 Site-directed mutagenesis of Aichi virus 3A identifies residues required for interaction with PI4KIII β and ACBD3. (A) Alanine scanning of individual and multiple residues in Aichi virus 3A was performed. Mutated Aichi virus 3A was transiently transfected into 293T cells and affinity purified using the Strep-Tag system. The eluate was blotted with anti-PI4KIII β , anti-ACBD3, and anti-Strep-Tag to normalize for bait expression. (B) Map of residues contributing to Aichi virus 3A and ACBD3 and PI4KIII β interaction. Residues labeled in red abolished myristoylation when converted to alanine. The predicted transmembrane region is highlighted in purple, and predicted alpha-helices are highlighted in teal. Black boxes indicate residues that reduce binding to ACBD3 or PI4KIII β by >90% when converted to alanine. Gray boxes indicate residues that do not change binding by >90%, and white boxes indicate residues for which no information is available.

virus, and bovine kobuvirus. The only exception was EV71, consistent with the reciprocal affinity purification experiments discussed above. Surprisingly, a band consistent with a complex containing Strep-Tag-ACBD3 and Aichi virus-3A-Flag was observed (Fig. 3, Aichi virus 3A lane) despite standard denaturing gel running conditions. Taken together, the results of this reciprocal affinity capture experiment imply direct interaction between ACBD3, PI4KIII β , and multiple picornaviruses.

Alignment of all Aichi virus 3A sequences in GenBank indicated >90% amino acid identity among the five sequences available, including 100% conservation in the N-terminal half of the protein.

Site-directed mutagenesis of Aichi virus 3A identifies residues required for interaction with PI4KIII β and ACBD3. To identify the critical residues for the interaction between Aichi virus 3A, PI4KIII β , and ACBD3, we employed alanine scanning of the 95-amino-acid Aichi virus 3A protein. In total, all 87 non-alanine residues were converted to alanine with a focus on single-site mutants on the N-terminal half, where all Aichi virus 3A sequences in GenBank are 100% conserved, in addition to multisite mutants on the C terminus (Fig. 4A). Of 87 positions mutated, approximately 20 residues (Fig. 4B), clustered at the N terminus,

severely reduced or abolished copurification of PI4KIII β (<10% of wild type, normalized to expression of the 3A protein in each experiment). In particular, the mutations R3A, I5A, NR2AA, NRVI2AAA, and NRVI2AAAA abolished PI4KIII β and ACBD3 interaction or reduced the amount by more than 90%. Although the R3A and G1A mutations both eliminate the N-terminal myristoylation of Aichi virus 3A, the lack of myristoylation does not account for the loss of PI4KIII β and ACBD3 interaction, since N2A, which also eliminates the N-terminal myristoylation, has no effect on copurification of either of these proteins (Fig. 4A). While 21 mutations could eliminate PI4KIII β association without affecting association of ACBD3 with Aichi virus 3A, all mutations that had a negative impact on ACBD3 association also severely reduced or eliminated PI4KIII β association. These results support the hypothesis that PI4KIII β association with Aichi virus 3A requires ACBD3 and also imply that 3A association with ACBD3 either enhances or stabilizes the interaction with PI4KIII β .

Chemical and genetic inhibition of PI4KIII β blocks Aichi virus replication. To assess whether the interaction between Aichi virus 3A and PI4KIII β was of functional importance for viral replication, we measured a *Renilla* luciferase replicon Aichi virus construct in the presence of chemical and genetic inhibition of

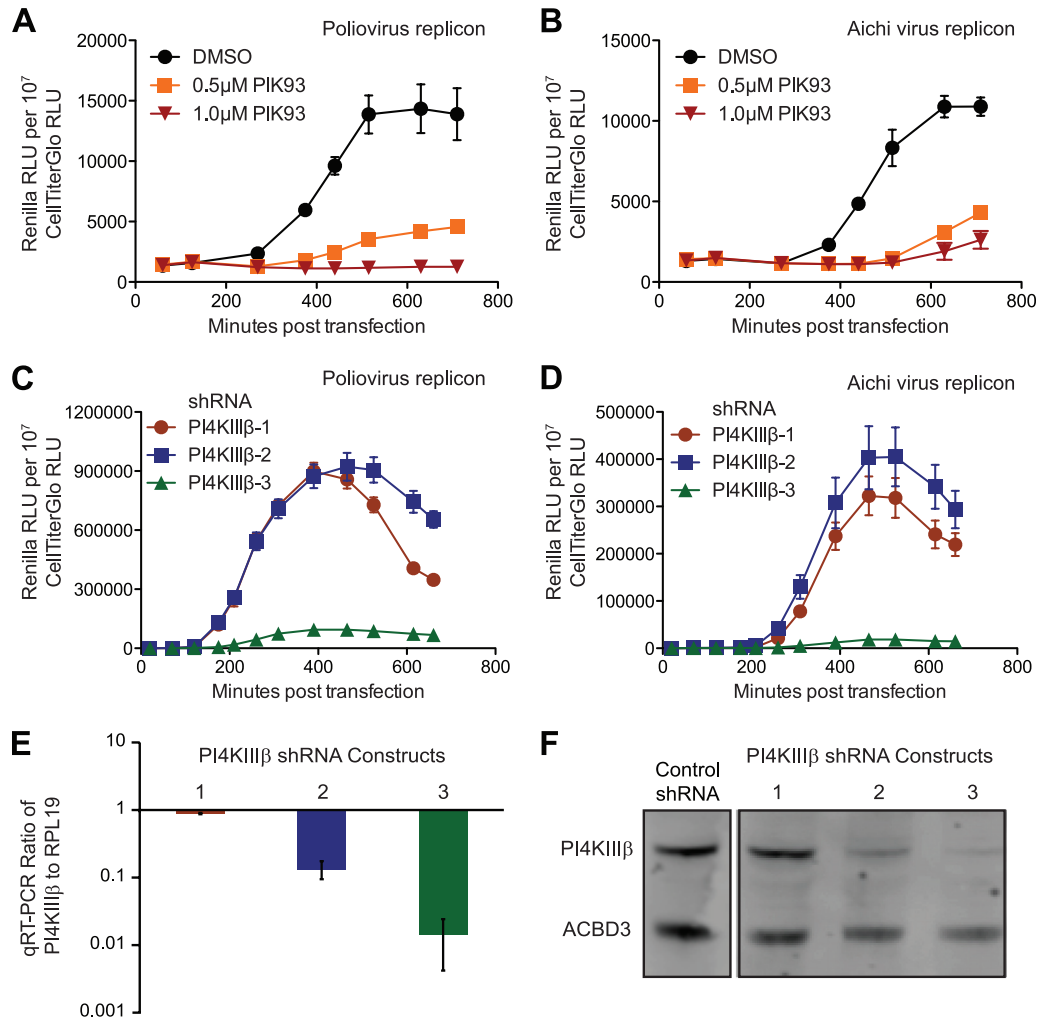


FIG 5 Chemical and genetic inhibition of PI4KIII β decreases Aichi virus replication. PIK93, an inhibitor of PI4KIII β , demonstrates a dose-dependent block in poliovirus (A) and Aichi virus replication (B). Genetic inhibition of PI4KIII β by stable shRNA blocks poliovirus (C) and Aichi virus replication (D) only when PI4KIII β levels are strongly reduced. RLU, relative light units. qRT-PCR (E) and Western blotting (F) of shRNA constructs demonstrate 10%, 90%, and 99% knockdown of PI4KIII β transcript levels and 0%, 60%, and 98% knockdown of PI4KIII β protein levels.

PI4KIII β . PIK93 is a small-molecule inhibitor of PI4K α and PI4KIII β (19). It has previously been shown to block the replication of poliovirus and hepatitis C virus replication with 50% effective concentrations (EC_{50}) of 0.14 and 1.9 μ M, respectively (1). The addition of 0.5 μ M and 1.0 μ M PIK93 resulted in a dose-dependent inhibition of Aichi virus replication similar to the dose-dependent decrease observed with poliovirus (Fig. 5A and B).

Stable shRNAs were used to reduce or nearly eliminate PI4KIII β expression in 293T cells, using previously published shRNAs (Fig. 5C and D) (4). PI4KIII β mRNA transcript abundance was reduced by up to 98% relative to the expression of the ribosome gene RPL19 (Fig. 5E). The shRNA-dependent knockdown PI4KIII β protein expression was also confirmed by Western blotting (Fig. 5F). Only the most potent shRNA construct inhibited Aichi virus and poliovirus replication, while incomplete knockdown of PI4KIII β did not significantly impact Aichi virus or poliovirus replication (Fig. 5C and D).

While siRNA knockdown of PI4KIII β completely abolished

Aichi virus and poliovirus replication, knockdown of ACBD3 demonstrated significantly reduced replication in both viruses (Fig. 6A and B). Although Aichi virus replication has been reported to be insensitive to brefeldin A (35), an inhibitor of GBF1/Arf1, we were surprised to find that siRNA knockdown of GBF1 also abolishes Aichi virus replication (Fig. 6B). Interestingly, the siRNA knockdown of GBF1 resulted in a loss of PI4KIII β , similar to what was achieved with a directed siRNA knockdown of PI4KIII β (Fig. 6C). While these results demonstrate a requirement for GBF1, it is possible that the replication defect is actually due to a loss of PI4KIII β indirectly caused by a loss of GBF1. These results support the hypothesis that the presence and activity of PI4KIII β are essential for Aichi virus replication, similar to what has been shown previously for poliovirus (17). These data also support the hypothesis that ACBD3 is functionally important for picornavirus replication, presumably by facilitating the interaction with PI4KIII β .

Reduced recruitment of PI4KIII β correlates with delayed or altered replication kinetics of Aichi virus replicons. To further

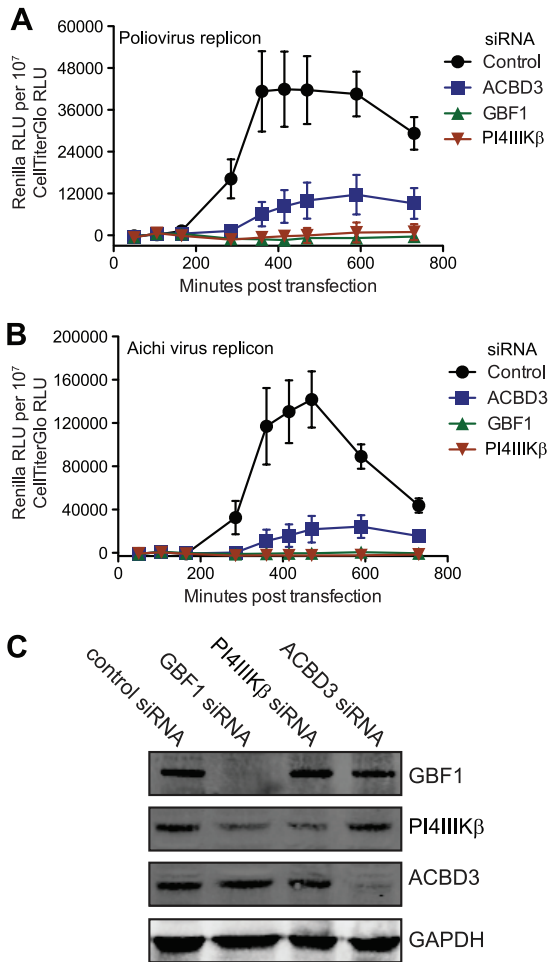


FIG 6 Genetic inhibition of physically interacting genes reduces viral replication. siRNA knockdown of GBF1, PI4KIII β , and ACBD3 reduces poliovirus (A) and Aichi virus (B) replicon growth in HeLa cells relative to control siRNA. RLU, relative light units. (C) Western blot of GBF1, PI4KIII β , ACBD3, and GAPDH in siRNA knockdown and control knockdown HeLa cells.

assess the requirement of the association between Aichi virus 3A and PI4KIII β for viral replication, we tested viral replication after replacing the wild-type 3A sequence with a series of point mutations based on our affinity purification results. No replication above background was measurable in the context of the Aichi virus replicon with the E11A mutation, which significantly disrupted copurification with PI4KIII β and reduced, but did not eliminate, ACBD3 association (Fig. 7A). Aichi virus with the G1A mutation also demonstrated no replication, though this is likely due to disruption of the P2-P3 3C proteolytic cleavage site. Myristoylation had a minimal impact on replication, as the N2A mutant had near-wild-type levels of replication, while replication of the R3A mutant was slightly delayed (Fig. 7B). Delayed replication was observed with the NR2AA, NRV2AAA, NRVI2AAAA, I5A, I12A, L20A, L21A, M24A, and HH26AA mutations, which significantly reduced copurification of PI4KIII β by >90%, though several of the mutations retained the same maximal level of replication as the wild-type Aichi virus replicon (Fig. 7C to F). The E22A mutation that retained wild-type PI4KIII β binding demonstrated replication kinetics that were slightly delayed but comparable to

those of the wild-type virus (Fig. 7G). The P59A mutation, which appeared to stimulate PI4KIII β copurification (Fig. 4A), resulted in a significant delay in replication, yet the virus continued to produce luciferase signal for more than 10 h posttransfection (Fig. 7G).

To ascertain whether the delayed replication in viruses with mutations in 3A that inhibited PI4KIII β association was due to the reduced PI4KIII β association, we examined the EC₅₀ of PIK93 in these mutant viruses compared to wild-type virus. PIK93 had an EC₅₀ of 0.60 μ M at 330 min posttransfection in the wild-type Aichi virus replicon. Interestingly, lower concentrations of PIK93 did not reduce the total amount of viral replication but only delayed viral replication in a dose-dependent fashion (Fig. 8A). However, the EC₅₀ of PIK93 was reduced more than 2-fold to 0.24 μ M in the I5A mutant and almost 20-fold to 0.03 μ M in the NRVI2AAAA mutant (Fig. 8B). These results support the notion that physical association of PI4KIII β with Aichi virus 3A via ACBD3 is required for replication.

DISCUSSION

In this study, we demonstrated that the 3A protein from multiple picornaviruses, including Aichi virus, bovine kobovirus, poliovirus, coxsackievirus B, and human rhinovirus 14, associates with PI4KIII β and the Golgi adaptor protein ACBD3. We note that some picornaviruses did not appear to associate with ACBD3. For example, we identified Rap1A as a candidate for interaction in the case of coronaviruses, and ACAD9 in the case of EV71. Previous studies have shown PI4KIII β is required for picornaviral replication and have created models of indirect recruitment of PI4KIII β by 3A (17). Through genetic and chemical inhibition, we demonstrated a requirement for PI4KIII β for Aichi virus replication, and we further defined the molecular determinants in Aichi virus 3A that are required for its physical association with ACBD3 and PI4KIII β .

ACBD3 is localized in the Golgi apparatus and contains an acyl-CoA binding domain, a putative nuclear localization signal, and a GOLD lipid trafficking domain (11). Mutation and overexpression of ACBD3 can cause disruption of the Golgi, implicating this protein in the maintenance of Golgi structure and function (36). It has also been implicated in a wide variety of cell signaling processes, from lipid transport to apoptosis. Though a genetic interaction with acyl-CoA binding protein and bromovirus replication has been demonstrated in *Saccharomyces cerevisiae*, our study is the first to demonstrate a physical interaction between a picornavirus and a host protein involved in acyl-CoA binding (20). The 3A protein of EV71, the one enteroviral 3A that did not strongly copurify with ACBD3, instead copurified with another acyl-CoA binding protein, acyl-CoA dehydrogenase family member 9 (ACAD9). These interactions suggest a potential mechanism for the localization of viral replication complexes to the endoplasmic reticulum-Golgi. It further suggests a role for lipid signaling and trafficking for the replication of picornaviruses. It is notable that despite alanine scanning of almost the entire Aichi virus 3A protein, only two mutations, R3A and I5A, were able to significantly disrupt association with ACBD3. In addition, affinity purification of ACBD3 in the presence of Aichi virus 3A revealed the presence of an SDS- and DTT-resistant Aichi virus 3A-ACBD3 complex, suggesting a highly stable interaction. Given the known roles of ACBD3 in Golgi structure and function, we hypothesize that this protein, in association with 3A, serves as a scaffold for

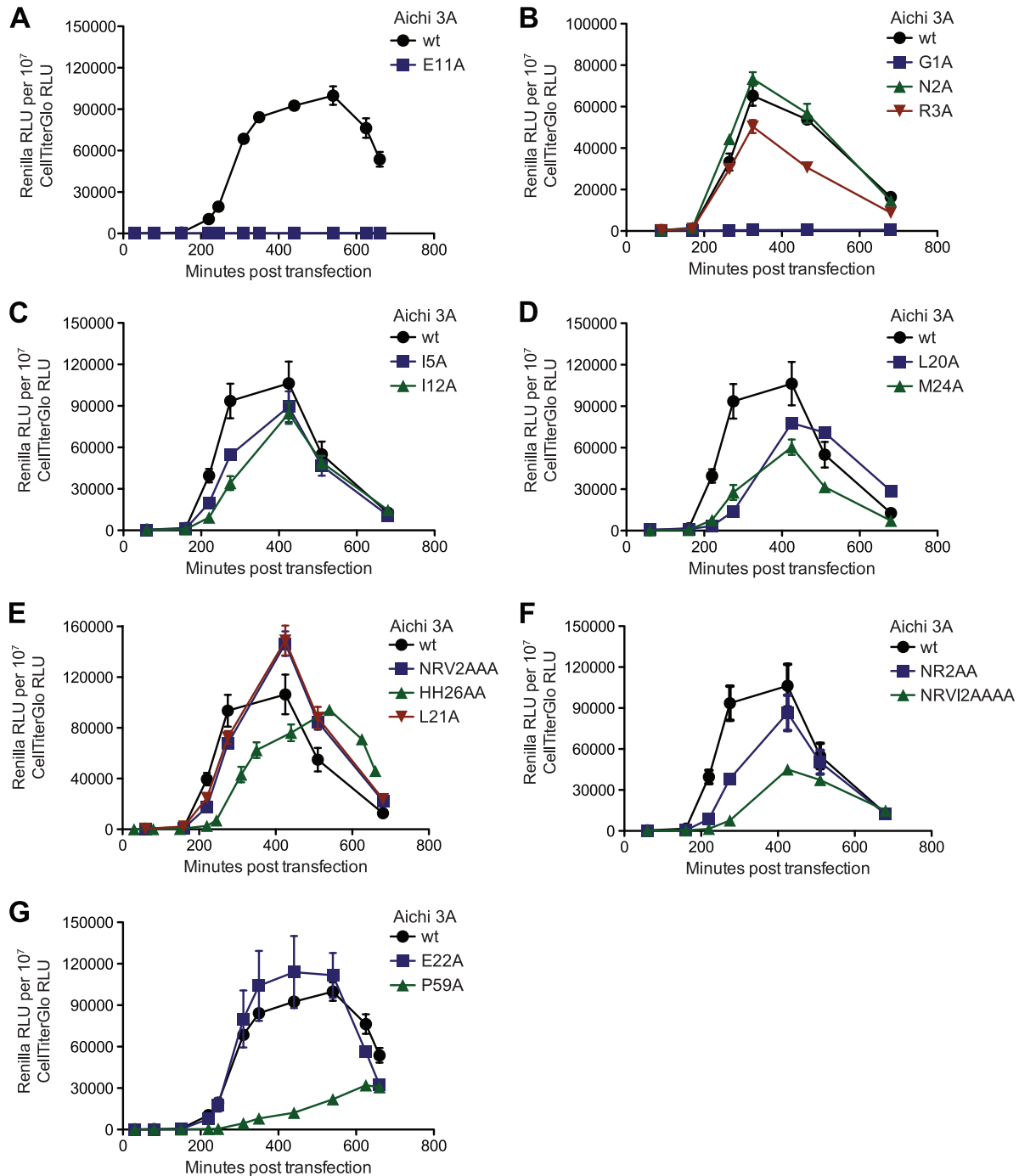


FIG 7 Reduced recruitment of PIKIII β correlates with delayed replication kinetics of Aichi virus replicons. 3A mutants were cloned into Aichi virus replicon and examined for replication efficiency. (A) The Aichi virus 3A E11A mutant is unable to replicate. (B) The Aichi virus 3A N2A and R3A mutants replicate, suggesting that myristoylation of Aichi virus 3A is not required for replication. (C to F) Aichi virus 3A mutants with mutations that reduce PI4KIII β binding by >90% are still capable of replicating, albeit with slower kinetics. (G) The Aichi virus 3A E22A mutant, which retains PI4KIII β and ACBD3 binding, demonstrates replication kinetics similar to those of wild-type, while the P59A mutant demonstrates significantly delayed and reduced replication kinetics. RLU, relative light units.

the generation of viral replication complexes and membrane remodeling.

The discovery of a myristoylation on Aichi virus, bovine kobuvirus, and klassevirus 3A is the first demonstration of myristoylation on a nonstructural picornavirus protein. The 3A protein is

myristoylated despite the presence of a noncanonical N-terminal sequence. Many viral proteins have previously been shown to be myristoylated, including retrovirus Gag protein, HIV Nef protein, hepadnavirus L protein, and arterivirus E protein, as well as the VP4 capsid protein of poliovirus and foot-and-mouth disease vi-

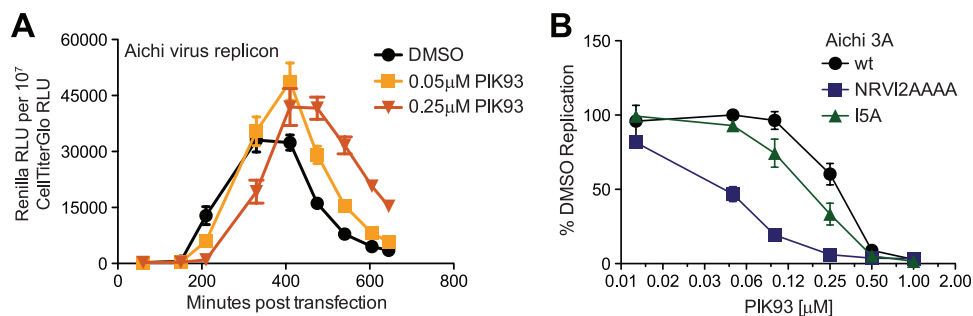


FIG 8 Aichi virus replicons with 3A mutants with reduced ability to recruit PI4KIII β are sensitized to PIK93 inhibition. (A) The EC₅₀ for PIK93 against wild-type Aichi virus replicon was 0.60 μ M at 330 min posttransfection. PIK93 inhibition of Aichi virus replication can be overcome at lower concentrations given a longer replication window. While 1 μ M and 0.5 μ M PIK93 significantly reduce and delay Aichi virus replication, lower concentrations of PIK93 merely delay replication of wild-type Aichi virus, though the virus eventually reaches the same level of replication. RLU, relative light units. (B) 3A mutants with significantly reduced binding of PI4KIII β are sensitized to PIK93 inhibition; the EC₅₀ is reduced to 0.24 μ M in the I5A mutant and to 0.03 μ M in the NRVI2AAAA mutant.

rus (28). The myristoylation on at least two different picornaviral proteins is significant, as it suggests a potential contributing mechanism for membrane association and reorganization, as well as a potential mechanism for concentrating picornaviral proteins and associating RNA replication with encapsidation. Nonetheless, we found that while myristoylation of 3A contributed somewhat to the binding of PI4KIII β , this posttranslational modification was not a determinant for binding of ACBD3. Interestingly, the binding of the cellular protein NCS-1 to PI4KIII β has been shown to be dependent on myristoylation of NCS-1 (17). It is plausible that myristoylation may serve as an enhancer of PI4KIII β activity or recruitment, but its precise role remains to be determined.

Our results also suggest that while inhibition of PI4KIII β may delay viral replication, even a very low affinity interaction between 3A, ACBD3, and PI4KIII β or partial activity of the enzyme may be sufficient to support viral replication. Indeed, mutants such as the L20A and M34A mutants, which do not affinity purify with PI4KIII β but retain their association with ACBD3, show only modest reductions in replication. Even more striking is the NRVI2AAAA mutant, which fails to recruit both PI4KIII β and ACBD3 and lacks the N-terminal myristoylation but still supports replication. Nevertheless, we have shown that the NRVI2AAAA mutant results in a 20-fold increase in sensitivity to chemical inhibition of PI4KIII β . This is similar to the effect observed in poliovirus, where the 3A2 mutant, which has a reduced ability to bind GBF1, replicates at near-wild-type levels and displays enhanced sensitivity to the GBF1 inhibitor brefeldin A (2). Furthermore, the ability to isolate 3A mutants that do not copurify with PI4KIII β suggests that 3A may influence ACBD3's ability to recruit PI4KIII β and is not merely limited to recruitment of a native ACBD3-PI4KIII β complex. From a therapeutic standpoint, our results suggest that chemical inhibitors that block association of PI4KIII β and viral 3A, or ACBD3 and viral 3A, would represent a complementary approach to simple inhibition of the kinase activity of PI4KIII β itself.

The investigation of multiple picornaviruses in our work demonstrates the broad importance of a common strategy for enterovirus and kobuvirus replication. Despite differences in their associations, such as enterovirus 3A binding of GBF1, viruses of these two genera ultimately operate through a common platform, the Golgi adaptor ACBD3, to recruit PI4KIII β .

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ADDENDUM

While this paper was under review, Sasaki et al. published a paper on the interaction of Aichi virus 3A with ACBD3 and PI4KIII β (35). The results of Sasaki et al. and our own are highly complementary, and the overall conclusions are consistent with respect to Aichi virus, despite the fact that the two studies used different experimental approaches.

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