The 3A Protein from Multiple Picornaviruses Utilizes the Golgi Adaptor Protein ACBD3 To Recruit PI4KIIIβ

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The activity of phosphatidylinositol 4-kinase class III beta (PI4KIIIβ) has been shown to be required for the replication of multiple picornaviruses; however, it is unclear whether a physical association between PI4KIIIβ 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 PI4KIIIβ 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 PI4KIIIβ. 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 picornaval 3A proteins and revealed the ability to bind PI4KIIIβ 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 PI4KIIIβ could be eliminated by mutation of specific residues, with little or no effect on recruitment of ACBD3. One mutation at the N terminus, I3A, significantly reduced copurification of both ACBD3 and PI4KIIIβ. The dependence of Aichi virus replication on the activity of PI4KIIIβ was confirmed by both chemical and genetic inhibition. Knockdown of ACBD3 by small interfering RNA (siRNA) also prevented replication of both Aichi virus and poliovirus. Point mutations in 3A that eliminate PI4KIIIβ association sensitized Aichi virus to PIK93, suggesting that disruption of the 3A/ACBD3/PI4KIIIβ 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 (PI4KIIIβ), 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 PI4KIIIβ 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 PI4KIIIβ 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 PI4KIIIβ and a Golgi adaptor...
protein, acyl-CoA binding domain protein 3 (ACBD3 or GCP60), under stringent capture and wash conditions. In the presence of methionine, N-terminal pyroglutamate from glutamine, loss of methionine, and carbamidomethylated cysteine were included as variable modifications. For database searching, peptide sequences were matched as tryptic peptides with no missed cleavages and with carbamidomethylated cysteine. Parent mass tolerance of 20 ppm on the LTQ-FT or 0.8 Da on the LTQ and LTQ-FT mass spectrometer (Thermo) equipped with a 10,000-lb/in² nano-trap quadrupole with Fourier transform ion cyclotron resonance (LTQ-FT) mass spectrometer (Thermo) equipped with an Ultimate high-performance liquid chromatography system (Waters) for reversed-phase chromatography with a C18 column (BEH130, 1.7-μm bead size, 100 μm 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-μm bead size, 100 μm 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-
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 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 15A mutant Aichi virus 3A (D); however, only the acetylated form was observed for the Aichi virus 3A R3A mutant (E).
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 in 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 additional posttranslational modifications were allowed, including 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 T7 amplified for 4 h, Turbo DNase cleaved for 15 min, and purified over a Zymo RNA-25 column (Zymo Research). One microgram of cut plasmid was T7 amplified for 4 h, Turbo DNase 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 cells were plated the night before in 100 μl complete medium per well in an opaque, white 96-well plate (Grenier Biotech; catalog no. 655075). Cells were transfected with 100 ng of T7-amplified RNA using a TransIT mRNA transfection kit (Mirus Bio) in 75 μl complete medium mixed with 1× 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 PI4KIIIβ 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 μg/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 μg 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, PI4KIIIβ, 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 μl of Dharmafect 1 transfection reagent and 125 μl total medium per well. At the same time, 60,000 log-phase HeLa cells were reverse wet transfected in 50 nM siRNA with 3 μl 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
membrane-reorganizing protein of picornviruses, protein 3A, we undertook an unbiased screen using single-step affinity purification of C-terminally Strept-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 3A of Aichi virus 3A, bovine kobuvirus 3A, and klassevirus 3A.

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

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<th>Source of bait 3A</th>
<th>Prey protein</th>
<th>Accession no.</th>
<th>Name or description</th>
<th>Mean z score</th>
<th>Replicate counts</th>
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d| 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 was the top two interacting proteins with Aichi virus 3A.

d| Values are reported as spectral counts, as described in Materials and Methods, where vertical bars delineate values from independent 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, cysteine carboxidomethylation, pyroglutamylation of glutamine, and oxidation, no other modifications were detected in these experiments.

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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 prey 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 PI4KIIIβ and ACBD3.** The top-ranked protein identified in affinity purifications with both Aichi virus 3A and bovine kobuvirus 3A was PI4KIIIβ (Table 1). PI4KIIIβ interaction with Aichi virus 3A and bovine kobuvirus 3A was confirmed by Western blot (Fig. 2A). Intriguingly, one peptide to PI4KIIIβ 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 PI4KIIIβ 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 Streptavidin beads (6). Using this more rapid procedure, PI4KIIIβ 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 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 Streptavidin 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 PI4KIIIβ 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 PI4KIIIβ 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 PI4KIIIβ 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 enterovirus and kobuviruses. In the absence of any transfected 3A proteins, PI4KIIIβ was found to copurify with ACBD3 (Fig. 3, empty vector lane), indicating that the interaction between ACBD3 and PI4KIIIβ does not require 3A. Affinity purification of ACBD3 in the presence of 3A proteins captured 3A from poliovirus, coxsackievirus B3, human rhinovirus 14, Aichi virus 14, and bovine kobuvirus 3A. Among these, in the presence of EV71 3A, ACBD3 copurifies with 3A proteins from multiple enteroviruses 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.
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, PI4KIIIβ, 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 PI4KIIIβ and ACBD3. To identify the critical residues for the interaction between Aichi virus 3A, PI4KIIIβ, and ACBD3, we employed alanine scanning of the 95-amino-acid Aichi virus 3A protein. In total, 87 nonalanine 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 PI4KIIIβ (<10% of wild type, normalized to expression of the 3A protein in each experiment). In particular, the mutations R3A, I5A, NR2AA, NRV2AAA, and NRVI2AAAA abolished PI4KIIIβ 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 PI4KIIIβ and ACBD3 association, 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 PI4KIIIβ 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 PI4KIIIβ association. These results support the hypothesis that PI4KIIIβ association with Aichi virus 3A requires ACBD3 and also imply that 3A association with ACBD3 either enhances or stabilizes the interaction with PI4KIIIβ.

Chemical and genetic inhibition of PI4KIIIβ blocks Aichi virus replication. To assess whether the interaction between Aichi virus 3A and PI4KIIIβ 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
PI4KIIIB. PIK93 is a small-molecule inhibitor of PI4Kα and PI4KIIIB (19). It has previously been shown to block the replication of poliovirus and hepatitis C virus replication with 50% effective concentrations (EC50) of 0.14 and 1.9 μM, respectively (1). The addition of 0.5 μM and 1.0 μM 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 PI4KIIIB expression in 293T cells, using previously published shRNAs (Fig. 5C and D) (4). PI4KIIIB mRNA transcript abundance was reduced by up to 98% relative to the expression of the ribosome gene RPL19 (Fig. 5E). The shRNA-dependent knockdown PI4KIIIB 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 PI4KIIIB did not significantly impact Aichi virus or poliovirus replication (Fig. 5C and D).

While siRNA knockdown of PI4KIIIB 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 PI4KIIIB, similar to what was achieved with a directed siRNA knockdown of PI4KIIIB (Fig. 6C). While these results demonstrate a requirement for GBF1, it is possible that the replication defect is actually due to a loss of PI4KIIIB indirectly caused by a loss of GBF1. These results support the hypothesis that the presence and activity of PI4KIIIB 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 PI4KIIIB.

Reduced recruitment of PI4KIIIB correlates with delayed or altered replication kinetics of Aichi virus replicons. To further
assess the requirement of the association between Aichi virus 3A and PI4KIIIβ 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 PI4KIIIβ 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, NRV12AAAA, 15A, 11A, L20A, L21A, M24A, and HH26AA mutations, which significantly reduced copurification of PI4KIIIβ 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 PI4KIIIβ 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 PI4KIIIβ 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 PI4KIIIβ association was due to the reduced PI4KIIIβ association, we examined the EC_{50} of PIK93 in these mutant viruses compared to wild-type virus. PIK93 had an EC_{50} of 0.60 μM 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 EC_{50} of PIK93 was reduced more than 2-fold to 0.24 μM in the I5A mutant and almost 20-fold to 0.03 μM in the NR12AAAA mutant (Fig. 8B). These results support the notion that physical association of PI4KIIIβ 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 kobuvirus, poliovirus, coxsackievirus B, and human rhinovirus 14, associates with PI4KIIIβ 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 cardioviruses, and ACAD9 in the case of EV71. Previous studies have shown PI4KIIIβ is required for picornaviral replication and have created models of indirect recruitment of PI4KIIIβ by 3A (17). Through genetic and chemical inhibition, we demonstrated a requirement for PI4KIIIβ 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 PI4KIIIβ.

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...
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-
rur (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 PI4KIIIβ, this posttranslational modification was not a determinant for binding of ACBD3. Interestingly, the binding of the cellular protein NCS-1 to PI4KIIIβ has been shown to be dependent on myristoylation of NCS-1 (17). It is plausible that myristoylation may serve as an enhancer of PI4KIIIβ activity or recruitment, but its precise role remains to be determined.

Our results also suggest that while inhibition of PI4KIIIβ may delay viral replication, even a very low affinity interaction between 3A, ACBD3, and PI4KIIIβ 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 PI4KIIIβ but retain their association with ACBD3, show only modest reductions in replication. Even more striking is the NRVI2AAAA mutant, which fails to recruit both PI4KIIIβ 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 PI4KIIIβ. This is similar to the effect observed in poliovirus, where the 3A2 mutant, which has a reduced ability to bind GBF1, replicates at near-null-type levels and displays enhanced sensitivity to the GBF1 inhibitor brefeldin A (2). Furthermore, the ability to isolate 3A mut atants that do not copurify with PI4KIIIβ suggests that 3A may influence ACBD3’s ability to recruit PI4KIIIβ and is not merely limited to recruitment of a native ACBD3-PI4KIIIβ complex. From a therapeutic standpoint, our results suggest that chemical inhibitors that block association of PI4KIIIβ and viral 3A, or ACBD3 and viral 3A, would represent a complementary approach to simple inhibition of the kinase activity of PI4KIIIβ 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 PI4KIIIβ.

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**ADDITIONAL**

While this paper was under review, Sasaki et al. published a paper on the interaction of Aichi virus 3A with ACBD3 and PI4KIIIβ (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.

**REFERENCES**


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