ACBD3 Interaction with TBC1 Domain 22 Protein Is Differentially Affected by Enteroviral and Kobuviral 3A Protein Binding

Alexander L. Greninger,¹,² Giselle M. Knudsen,³ Miguel Betegon,¹,² Alma L. Burlingame,³ Joseph L. DeRisi¹,²
Howard Hughes Medical Institute, San Francisco, California, USA; Department of Biochemistry and Biophysics, UCSF, San Francisco, California, USA; Department of Pharmaceutical Chemistry, UCSF, San Francisco, California, USA

ABSTRACT Despite wide sequence divergence, multiple picornaviruses use the Golgi adaptor acyl coenzyme A (acyl-CoA) binding domain protein 3 (ACBD3/GCP60) to recruit phosphatidylinositol 4-kinase class III beta (PI4KB), a factor required for viral replication. The molecular basis of this convergent interaction and the cellular function of ACBD3 are not fully understood. Using affinity purification-mass spectrometry, we identified the putative Rab33 GTPase-activating proteins TBC1D22A and TBC1D22B as ACBD3-interacting factors. Fine-scale mapping of binding determinants within ACBD3 revealed that the interaction domains for TBC1D22A/B and PI4KB are identical. Affinity purification confirmed that PI4KB and TBC1D22A/B interactions with ACBD3 are mutually exclusive, suggesting a possible regulatory mechanism for recruitment of PI4KB. The C-terminal Golgi dynamics (GOLD) domain of ACBD3 has been previously shown to bind the 3A replication protein from Aichi virus. We find that the 3A proteins from several additional picornaviruses, including hepatitis A virus, human parechovirus 1, and human klassevirus, demonstrate an interaction with ACBD3 by mammalian two-hybrid assay; however, we also find that the enterovirus and kobuvirus 3A interactions with ACBD3 are functionally distinct with respect to TBC1D22A/B and PI4KB recruitment. These data reinforce the notion that ACBD3 organizes numerous cellular functionalities and that RNA virus replication proteins likely modulate these interactions by more than one mechanism.

IMPORTANCE Multiple viruses use the same Golgi protein (ACBD3) to recruit the lipid kinase phosphatidylinositol 4-kinase class III beta (PI4KB) in order to replicate. We identify a new binding partner of ACBD3 in the evolutionarily conserved Rab GTPase-activating proteins (RabGAPs) TBC1D22A and -B. Interestingly, TBC1D22A directly competes with PI4KB for binding to the same location of ACBD3 by utilizing a similar binding domain. Different viruses are able to influence this interaction through distinct mechanisms to promote the association of PI4KB with ACBD3. This work informs our knowledge of both the physical interactions of the proteins that help maintain metazoan Golgi structure and how viruses subvert these evolutionarily conserved interactions for their own purposes.

RESEARCH ARTICLE

Photonaviruses are single-stranded positive-sense RNA viruses that replicate on intracellular membranes (1). Previous work has indicated that the 3A protein is responsible for the reorganization of Golgi membranes to create viral replication organelles (2, 3). The molecular basis of this reorganization has only recently become apparent through the dual discoveries that enteroviral 3A directly interacts with Golgi-specific brefeldin A resistance guanine nucleotide exchange factor 1 (GBF1) and that many RNA viruses require the activity of phosphatidylinositol 4-kinase class III beta (PI4KB) for viral replication (2, 3). The picornavirus 3A-ACBD3 interaction is required for replication (4). Two protein-protein interaction screens recently demonstrated that the 3A proteins of enteroviruses and kobuviruses both interact with the host Golgi adaptor protein acyl coenzyme A (acyl-CoA) binding domain protein 3 (ACBD3/GCP60) to recruit PI4KB to viral replication organelles (5, 6). ACBD3 is a highly conserved Golgi complex-associated 60-kDa protein among metazoans that contains a remarkably long N-terminal acyl-CoA binding domain, a coiled-coil domain composed of a charged amino acid region (CAR) and glutamine-rich region (Q-rich), and a highly conserved C-terminal Golgi dynamics domain (GOLD domain) that interacts with the Golgi resident protein giantin/GOLGB1 (7). The level of expression of ACBD3 has been shown to be important for the maintenance of the Golgi structure (7). The picornavirus 3A-ACBD3 interaction is required for replication, as knockdown of ACBD3 significantly reduces poliovirus and Aichi virus replication (5, 6). Furthermore, mutations that reduce binding of Aichi virus 3A to ACBD3 sensitize virus to PI4KB inhibitors, suggesting that recruitment of PI4KB is mediated by the 3A-ACBD3 interaction (5). Intriguingly, a recent yeast two-hybrid screen of hantavirus nonstructural proteins also dem-
onstrated a physical interaction with ACBD3, suggesting that this protein may be broadly required for viral replication due to its native association with PI4KB (8). Further study of the cellular function of ACBD3 is warranted to better understand its role in viral replication and its potential as a target for therapeutic intervention.

In this study, we used affinity purification-mass spectrometry (AP-MS) to identify new protein-protein interactions of ACBD3. We discovered a new interaction with the putative Rab33 GTPase-activating protein (GAP) TBC1D22A/B (9). Detailed mapping of the ACBD3 interactome revealed a mutually exclusive interaction between PI4KB and TBC1D22A/B for a highly conserved region in the coiled-coil domain of ACBD3. While picornaviral 3A proteins bind to the C-terminal GOLD domain on ACBD3, we also find that the enterovirus and kobuvirus 3A proteins interact with ACBD3 in a functionally distinct manner based on differential displacement of TBC1D22A/B, isolation of an ACBD3 mutant that displays differential binding between the 3A proteins, and different ACBD3 binding sites on the 3A proteins.

RESULTS

AP-MS of Strep-tagged ACBD3 reveals interaction with TBC1D22A/B. Based on its identification as a specific protein interaction partner for picornavirus 3A proteins, the multifunctional protein ACBD3 was hypothesized to be used by 3A as an organizing-scaffold protein at the Golgi interface. To identify potential novel interactions of ACBD3 relevant to viral replication and its potential as a target for therapeutic intervention, reciprocal affinity purification was undertaken with N- and C-terminally Strep-tagged ACBD3 in 293T cells and identified interacting proteins by mass spectrometry (Table 1; see also Tables S1, Tab S1.3A and S1.3B in the supplemental material). Non-specific interacting proteins were defined as the highest-frequency proteins identified in a background model of 550 AP-MS data sets compiled from the structural and nonstructural genes from 12 different picornaviruses, excluding the 3A protein itself (see Table S1, Tab S1.1).

Using this AP-MS approach combined with Z score ranking for interaction specificity, we found that ACBD3 interacted with the TBC1 domain family member 22A protein (TBC1D22A) in a highly specific manner and at levels that were comparable to those of PI4KB (Table 1; full Z scores are provided in Table S1, Tab S1.4 in the supplemental material). Additional proteins such as transmembrane protein 55B isoform 2 (TMEM55B) and protein phosphatase 1H (PPM1H) were also observed to be specific for ACBD3. The known ACBD3-interacting Golgi protein giantin/GOLGB1 was identified in affinity purifications of N-terminally tagged ACBD3 only in a buffer that included potassium chloride and divalent cations (Table 1) (7).

TBC1D22A is a 58-kDa protein that is localized to the Golgi apparatus and involved in Golgi membrane maintenance, along with its closely related isoform TBC1D22B (10). Both contain a C-terminal TBC domain that is responsible for Rab GTPase activation with a putative preference for Rab33A/B (9). Overexpression of TBC1D22B was shown to cause disruption of the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC), which was dependent on its GTPase-activating protein (GAP) activity (10). Based on the localization of ACBD3 and picornaviral replication to ERGIC-associated membranes, TBC1D22A/B merited further investigation.

Affinity purification of TBC1D22A/B or PI4KB captures ACBD3 and 14-3-3 proteins. To confirm the ACBD3-TBC1D22A interaction, reciprocal affinity purification was undertaken with N- and C-terminally Strep-tagged TBC1D22A and TBC1D22B (see Table S1, Tab S1.3A and S1.3B in the supplemental material). The top-ranking interacting protein for both TBC1D22A and TBC1D22B was ACBD3 (Table 1). Multiple 14-3-3 isoforms were also found in high abundance in affinity purifications for both TBC1D22A and TBC1D22B (see Table S1, Tab S1.5). 14-3-3 proteins are 27-kDa adaptor proteins that bind phosphoserine residues on a multitude of cellular proteins involved in diverse signaling pathways (11). TBC1D22A/B phosphopeptides are reported in Table S1, Tab S1.6 and include a putative 14-3-3 recognition site at serine 167 (TBC1D22A) or serine 154 (TBC1D22B; spectra are provided in Fig. S1). No peptides for PI4KB were recovered in any AP-MS experiments on TBC1D22A or TBC1D22B, suggesting that ACBD3-TBC1D22A/B-containing complexes do not contain PI4KB.

To further confirm the PI4KB-ACBD3 interaction, affinity purification of Strep-tagged PI4KB was performed. The top-ranking protein was ACBD3, while a 14-3-3 isoform was ranked third (Table 1; see also Table S1, Tab S1.3D in the supplemental material). The 14-3-3 proteins have been shown to bind PRKD1-phosphorylated serine 294 of PI4KB and influence its catalytic activity (12). This phosphorylated site and others were identified in our affinity purifications as well, as reported in Table S1, Tab S1.6 (spectrum is provided in Fig. S1). Several additional proteins of unknown significance (C10orf76, GBA, and MTA2) discovered in a recent Flag tag AP-MS experiment on PI4KB also ranked highly in our screen (13). Finally, as in the reciprocal case above, no peptides for TBC1D22A or TBC1D22B were recovered in any AP-MS experiments on PI4KB.

ACBD3-TBC1D22A/B interaction maps to coiled-coil region and overlaps with PI4KB-interacting region of ACBD3. To further query the hypothesis that TBC1D22A/B binding and PI4KB binding may represent distinct, potentially competitive binding states of ACBD3, a mammalian two-hybrid reporter assay was used to map the sites of interaction on ACBD3 by deletion mutagenesis (Fig. 1). N-terminal truncations of the acyl-CoA binding domain, C-terminal truncations of the GOLD domain, and alanine scanning through the entirety of the CAR domain did not affect ACBD3 binding to TBC1D22A/B, while deletions and point mutations located in the conserved glutamine-rich coiled-coil region between residues 246 and 321 significantly reduced TBC1D22A/B binding (see Fig. S2 and S3 in the supplemental material; also Fig. 1). This region has previously been implicated in binding PI4KB (6). Mapping of PI4KB on ACBD3 revealed significant overlap with the region bound by TBC1D22A/B (Fig. 1A). Three mutants with mutations in the glutamine-rich region (VQF255AAA, PGN267AAA, and EQHY281AAAA) significantly reduced ACBD3 binding to both PI4KB and TBC1D22A/B (Fig. 1B; see also Fig. S2B). However, we could isolate no mutant of ACBD3 that retained wild-type levels of PI4KB binding while disrupting TBC1D22A/B binding, or vice versa, including mutation of a recovered phosphorylation site, SS344 (see Table S1, Tab S1.6). Two single point mutants in the glutamine-rich region (F258A and Y285A) did demonstrate a significant difference in PI4KB binding versus TBC1D22B and merit further investigation (Fig. S5). Although we have mapped the critical binding region of TBC1D22A/B to the Q-rich region of ACBD3, we cannot discount
protein AP-MS experiments, excluding the 3A protein itself (Materials and Methods). Shown here are the top-scoring proteins that appeared in at least two replicate experiments.

### TABLE 1 Interacting proteins identified by AP-MS for ACBD3, TBC1D22A, TBC1D22B, and PI4KIIIb

| Bait   | Accession no. | Gene designation | Protein name                                      | Z score | Replicate count sum |
|--------|----------------|------------------|--------------------------------------------------|---------|---------------------|
| ACBD3 NS | 18528652       | ACBD3            | Golgi resident protein GCP60                     | 23.5    | 699                 |
| ACBD3 NS | 22507409       | TBC1D22A         | TBC1 domain family member 22A                    | 23.5    | 13                  |
| ACBD3 NS | 15481684       | TMEM55B          | Transmembrane protein 55B isoform 2             | 19.5    | 11                  |
| ACBD3 NS | 311771621      | PI4KB            | Phosphatidylinositol 4-kinase beta isoform 2     | 15.6    | 5                   |
| ACBD3 CS | 15828652       | ACBD3            | Golgi resident protein GCP60                     | 23.5    | 587                 |
| ACBD3 CS | 15481684       | TMEM55B          | Transmembrane protein 55B isoform 2             | 19.5    | 6                   |
| ACBD3 CS | 149944715      | PPM1H            | Protein phosphatase 1H                           | 15.6    | 14                  |
| ACBD3 CS | 22507409       | TBC1D22A         | TBC1 domain family member 22A                    | 11.7    | 12                  |
| ACBD3 NS | 311771621      | PI4KB            | Phosphatidylinositol 4-kinase beta isoform 2     | 7.8     | 6                   |
| ACBD3 CS | 15828652       | ACBD3            | Golgi resident protein GCP60                     | 23.5    | 701                 |
| ACBD3 CS | 148569894      | GOLGB1           | Golgin subfamily B member 1                      | 23.5    | 62                  |
| ACBD3 CS | 83641874       | CPVL             | Probable serine carboxypeptidase CPVL precursor  | 23.5    | 21                  |
| ACBD3 CS | 15481684       | TMEM55B          | Transmembrane protein 55B isoform 2             | 23.5    | 17                  |
| ACBD3 CS | 11386135       | BCKDHA           | 2-Oxoisovalerate dehydrogenase subunit alpha, mitochondrial isoform 1 precursor | 15.6 | 27 |
| ACBD3 CS | 34101272       | BCKDHB           | 2-Oxoisovalerate dehydrogenase subunit beta, mitochondrial isoform 1 precursor | 15.6 | 13 |
| ACBD3 NS | 38026892       | ALG6             | Dolichyl pyrophosphate Man9GlcNAc2 alpha-1,3-glucoyltransferase precursor | 15.6 | 12 |
| ACBD3 CS | 19923748       | DLST             | Dihydrolipoyllysine residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial isoform 1 precursor | 15.6 | 8 |

### Interacting proteins identified by AP-MS for ACBD3, TBC1D22A, TBC1D22B, and PI4KIIIb

| Accession no. | Gene designation | Protein name                                      | Z score | Replicate count sum |
|---------------|------------------|--------------------------------------------------|---------|---------------------|
| 124494254     | PA2G4            | Proliferation-associated protein 2G4              | 11.7    | 7                   |
| 7019485       | PDCD6            | Programmed cell death protein 6                   | 11.7    | 7                   |
| 311771621     | PI4KB            | Phosphatidylinositol 4-kinase beta isoform 2      | 11.7    | 6                   |
| 6912582       | PEF1             | Peflin                                           | 11.7    | 6                   |
| 5571641       | KIDINS220        | Kinase D-interacting substrate of 220 kDa         | 7.8     | 4                   |
| 38679884      | SRI              | Sorcin isoform b                                 | 7.3     | 1                   |
| 15828652      | ACBD3            | Golgi resident protein GCP60                     | 23.5    | 42                  |
| 83641874      | CPVL             | Probable serine carboxypeptidase CPVL precursor   | 23.5    | 24                  |
| 149944715     | PPM1H            | Protein phosphatase 1H                            | 23.5    | 16                  |
| 311771621     | PI4KB            | Phosphatidylinositol 4-kinase beta isoform 2      | 19.5    | 18                  |
| 15481684      | TMEM55B          | Transmembrane protein 55B isoform 2              | 19.5    | 11                  |
| 4504805       | BLZF1            | Golgin-45                                        | 15.6    | 14                  |
| 38026892      | ALG6             | Dolichyl pyrophosphate Man9GlcNAc2 alpha-1,3-glucoyltransferase precursor | 11.7 | 12 |
| 22507409      | TBC1D22A         | TBC1 domain family member 22A                    | 11.7    | 7                   |
| 19923748      | DLST             | Dihydrolipoyllysine residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial isoform 1 precursor | 7.8 | 9 |

**a** Interacting proteins identified by AP-MS for ACBD3, TBC1D22A, TBC1D22B, and PI4KIIIb were weighted by Z score of the peptide counts. Proteins are listed here with replicate Z scores and peptide counts in the experimental set with a minimum of n = 5 biological replicates and were scored against a background set of 550 unrelated picornaviral protein AP-MS experiments, excluding the 3A protein itself (Materials and Methods). Shown here are the top-scoring proteins that appeared in at least two replicate experiments and with >1 count in at least one experiment; a full Z score table is provided in Table S1, Tab SI.4 in the supplemental material. The major interacting proteins for ACBD3 included TBC1D22A and PI4KB, as well as PPM1H, TMEM55B isoform 2, CPVL, and GOLGB1. Reciprocal AP-MS experiments with TBC1D22A, its closely related isoform TBC1D22B, and PI4KB confirmed interaction with ACBD3. Proteins are C-terminally StrepII-tagged (CS) unless otherwise noted as N-terminally tagged (NS).
the role of the GOLD domain, as it appears to have an influence on the binding of these proteins as shown in Fig. 1A. We also note that TBC1D22A/B was not detected in any PI4KB AP-MS experiment nor was PI4KB detected in any TBC1D22A/B AP-MS experiment (see Table S1, Tab S1.3B–S1.3D). Together, these data support the notion that PI4KB and TBC1D22A/B participate in a mutually exclusive relationship with ACBD3.

The N terminus of TBC1D22A/B is required for ACBD3 interaction. To understand whether the RabGAP domain or some other region of TBC1D22A/B was responsible for the interaction with ACBD3, both mammalian two-hybrid mapping and AP-Western blotting were performed on deletion mutants of our lab (spectra are provided in Fig. S1).

Deletion mapping, alanine mutagenesis, and AP-Western blotting of TBC1D22A revealed a significant involvement of serines 165 and 167 for 14-3-3 protein recruitment. This site matches the canonical type I 14-3-3 binding motif of R-[SFYW]-X-pS- and point mutants that disrupted 14-3-3 binding retained the ability to bind ACBD3, while the TBC1D22A VL100AA mutant that disrupted ACBD3 binding was still able to bind 14-3-3 isoforms, similarly to wild-type TBC1D22A (Fig. 2A; see also Fig. S4B in the supplemental material). While the significance of TBC1D22A/B is thus far not known, the possibility that disruption of ACBD3 interaction with TBC1D22A/B might allow an alternative recruitment mechanism for 14-3-3 should be considered. Deletion mapping of TBC1D22A/B similarly localized the ACBD3 interaction to a predicted alpha-helix with conserved sequence to TBC1D22A, including the valine-leucine pair (Fig. 2C; see also Fig. S4C in the supplemental material). Alamine scanning of this region on TBC1D22B also revealed substantial defects (∼100-fold decrease) in binding, especially at V1100 (Fig. 2D). Mutation of phosphorylation sites on TBC1D22B discovered by mass spectrometry, all of which reside outside the identified interacting region, to glutamic acid as a phosphomimetic did not affect binding to ACBD3 (see Fig. S4D and Table S1, Tab S1.6). Mammalian two-hybrid mapping on TBC1D22A/B was confirmed by AP-Western blotting and AP-MS (see Fig. S4A to C). Interestingly, six total AP-MS experiments with full-length TBC1D22A and TBC1D22B and deletion mutants of TBC1D22A, all of which retained ACBD3 binding, showed that they copurified with ARFGEF1, a trans-Golgi membrane-localized Sec7 domain containing the guanine exchange factor for Arf1 (13). This interaction is highly specific, as peptides to ARFGEF1 were detected in only those six deletion and full-length TBC1D22A/B AP-MS experiments out of a total of >2,100 AP-MS runs in our lab (spectra are provided in Fig. S1).

FIG 1 TBC1D22A/B interaction on ACBD3 is localized to the coiled-coil region and overlaps the PI4KB-interacting region. (A) Mapping of TBC1D22A/B and PI4KB binding localizes to the glutamine-rich (Q) region on ACBD3 by mammalian two-hybrid screening. The three proteins demonstrate similar binding values for all of the mutants tested, with a slight preference of TBC1D22A over TBC1D22B and PI4KB. (B) Alanine mutants across the C-terminal half of ACBD3 demonstrate that TBC1D22A/B and PI4KB binding to ACBD3 is disrupted only by mutations in the glutamine-rich region and not by mutations in the C-terminal GOLD domain. Binding values for the protein-protein interaction reporter (firefly luciferase) are plotted as a percentage of the transfection control values. The critical region defined by the collection of constructs is demarcated by the yellow box with the dashed outline (ACBD, acyl-CoA binding domain; CAR, charged amino acid region; Q, glutamine-rich region; GOLD, Golgi dynamics domain).
Interaction with 14-3-3 proteins remains unknown, these data suggest that TBC1D22A interaction with ACBD3 is not mediated by 14-3-3 isoforms or vice versa.

PI4KB-ACBD3 interaction maps to the N terminus of PI4KB. We employed both AP-Western blotting and mammalian two-hybrid mapping in conjunction with deletion mutants to localize the region of PI4KB required for interaction with ACBD3 (Fig. 3). By N-terminal and C-terminal deletion, a region of 19 amino acids, extending from residues 53 to 70, of PI4KB was necessary for interaction with ACBD3 by both assays, indicating that the catalytic domain, the lipid kinase unique (LKU) region, and the Hom2 region of PI4KB were dispensable (16) (Fig. 3A; see also Fig. S5A, B, and C in the supplemental material). Analogously to what was found in TBC1D22A/B, alanine scanning of this narrow region in PI4KB revealed that a valine-leucine pair (VL67) was required for ACBD3 interaction with additional contribution from upstream residues (Fig. 3B), suggesting that these two proteins bind ACBD3 via similar motifs. These results were also confirmed by AP-Western blotting (see Fig. S5A to C). Two mutants, VL67 and IDP55, that retained the ability to bind alternative PI4KB partners, C10orf76 and Rab11B, were identified, suggesting that the ACBD3 binding deficit was specific (Fig. 3B). We note that the QE65AA mutant demonstrated no ability to bind C10orf76 or Rab11B but bound to ACBD3 at levels 6-fold greater than those for the wild type according to the mammalian two-hybrid reporter (Fig. 3B), although it is not necessarily clear whether these differences in binding are due to increased binding to one factor or to an inability to bind another competing factor. Finally, mutation of PI4KB phosphorylation sites discovered by mass spectrometry, all of which reside outside the critical interaction region, to glutamic acid did not affect binding to ACBD3, C10orf76, or Rab11B (see Fig. S5D).

TBC1D22A/B competes with PI4KB for ACBD3 binding in vitro. The combined AP-MS and fine-scale mapping data for TBC1D22A/B and PI4KB suggest a mutually exclusive binding relationship with ACBD3. If this were the case, we would expect there to be a competitive binding relationship between these two proteins and ACBD3. To qualitatively test this hypothesis in a competition experiment, increasing amounts of protein lysate from 293T cells expressing TBC1D22A-Flag were added to premixed 293 lysate from PI4KB-V5- and ACBD3-Strep-expressing cells (Fig. 4A). Affinity purification of ACBD3-Strep revealed decreasing amounts of copurified PI4KB-V5 as a function of increasing amounts of TBC1D22A-Flag (Fig. 4B). This competition was independent of RabGAP activity, as only the N terminus of the mammalian two-hybrid reporter (Fig. 3B), although it is not necessarily clear whether these differences in binding are due to increased binding to one factor or to an inability to bind another competing factor. Finally, mutation of PI4KB phosphorylation sites discovered by mass spectrometry, all of which reside outside the critical interaction region, to glutamic acid did not affect binding to ACBD3, C10orf76, or Rab11B (see Fig. S5D).

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TBC1D22A was required (Fig. 4C). 293T cell lysate that did not express TBC1D22A-Flag could not compete off PI4KB-V5 (Fig. 4D).

3A binding region of ACBD3 localizes to the GOLD domain and associates with ACBD3 self-binding. Previously, we and others demonstrated that enteroviral and kobuviral 3A proteins interact with ACBD3 by AP-MS and AP-Western blotting (5, 6). To test whether other picornavirus 3A proteins bound ACBD3 more transiently, we tested hepatovirus, klassevirus, and parechovirus 3A proteins in the mammalian two-hybrid assay. In this assay, both kobuvirus and enterovirus 3A demonstrated a significant contribution of residues VL67 and IDP55 to its interaction with ACBD3. Binding of PI4KB interactors Rab11B and C10orf76 was used to control for global protein defects. Mutant QE65AA abrogated binding of PI4KB to ACBD3 self-binding.

FIG 3 ACBD3 interaction localizes to the N terminus on PI4KB and is disrupted by the same valine-leucine mutation. (A) Deletion mapping of PI4KB localizes its interaction with ACBD3 to the far N terminus on PI4KB by mammalian 2-hybrid screening. (B) Alanine scanning of the putative alpha-helix on PI4KB demonstrated a significant contribution of residues VL67 and the far N terminus on PI4KB by mammalian 2-hybrid screening. (B) Alanine scanning of the putative alpha-helix on PI4KB demonstrated a significant contribution of residues VL67 and the far N terminus on PI4KB by mammalian 2-hybrid screening.
much of the N-terminal half of poliovirus 3A revealed several point mutants C terminal to the GBF1 binding site that were critical for binding ACBD3 by AP-Western blotting (D29A, R34A, KKGW42-KGA, and R54A) (see Fig. S9C in the supplemental material). These results implicate multiple portions of Aichi virus 3A for binding to ACBD3, while poliovirus 3A appears to contain a defined ACBD3 binding domain that is C-terminal to its GBF1 binding region and dimerization region (20).

Kobuviral 3A proteins prevent TBC1D22A binding to ACBD3, while enteroviral 3A proteins do not. Given that TBC1D22A/B appears to compete with PI4KB for ACBD3 binding and that picornaviruses are dependent on PI4KB for activity, we hypothesized that picornavirus 3A interaction with ACBD3 could influence the outcome of this competition. We note that in the picornavirus 3A AP-MS data that we have published previously (5), peptides to TBC1D22A/B were recovered in multiple enteroviral 3A AP-MS experiments while no peptides to TBC1D22A/B were ever identified in kobuviral 3A AP-MS experiments where a stable ACBD3-PI4KB complex was identified (see Table S1, Tab SI.4 in the supplemental material) (5). To test this hypothesis, we examined whether the presence of different 3A proteins affected the TBC1D22A-ACBD3 interaction. We coexpressed kobuvirus 3A and enterovirus 3A proteins together with TBC1D22A and then assayed affinity-purified TBC1D22A by Western blotting. Whereas the expression of enterovirus 3A proteins did not affect the recovery of affinity-purified TBC1D22A, we observed an apparent effect on the expression of TBC1D22A in the presence of kobuvirus 3A (see Fig. S7).

We investigated whether the presence of Aichi virus 3A or poliovirus 3A could affect the endogenous level of TBC1D22A mRNA. Using HEK293T cells transfected with Aichi virus 3A-Flag or poliovirus 3A-Flag, we measured the level of TBC1D22A mRNA by quantitative reverse transcription-PCR (qRT-PCR). There was no significant difference in the level of TBC1D22A mRNA in the presence of poliovirus 3A and only modest changes (<14%) in the presence of TBC1D22A (see Fig. S8 in the supplemental material).

To investigate the effect of picornavirus 3A protein interaction with respect to PI4KB and TBC1D22A interaction with ACBD3, and to avoid the apparent effect of coexpression differences in the presence of 3A proteins, we separated the expression of the proteins by independently transfecting Flag-tagged picornavirus 3A proteins and TBC1D22A-Flag into different plates of HEK293T cells. We then immunoprecipitated TBC1D22A-Flag complexes onto beads and added exogenous HEK293T lysates containing overexpressed poliovirus 3A-Flag and Aichi virus 3A-Flag. Finally, the TBC1D22A-Flag beads were recovered and assayed for ACBD3 and 3A-Flag recruitment by Western blotting (Fig. 7A). Poliovirus 3A-Flag could be readily detected in TBC1D22A-ACBD3-containing complexes, while Aichi virus 3A-Flag could not (Fig. 7B). Quantification of this difference demonstrated a >7-fold increase in poliovirus 3A recovery from the TBC1D22A-Flag beads over that of Aichi virus 3A (Fig. 7C). Collectively, these results support a model whereby the kobuviral and enteroviral 3A proteins may differentially modulate the interaction of PI4KB and TBC1D22A/B with ACBD3.

**DISCUSSION**

In this study, we have identified TBC1D22A/B as a new interacting partner of ACBD3, a protein of central importance in Golgi organization and picornaviral replication. TBC1D22A/B is a Golgi membrane-localized putative Rab33 RabGAP (9). Altered ER-Golgi morphology has been associated with overexpression of TBC1D22B and is dependent on the presence of TBC1D22B RabGAP activity (10). We found that the interaction between TBC1D22A/B and ACBD3 was dependent on a narrow region that maps to a predicted helix in the N terminus of TBC1D22A/B. This interaction may determine localization of these RabGAPs to the Golgi membrane and their ability to affect Golgi morphology. TBC1D22A/B bound to the same region on ACBD3 as did PI4KB, and we found that the same residues that were critical for TBC1D22A/B binding were also critical for PI4KB binding. Together with our finding that TBC1D22A/B directly competed with PI4KB for ACBD3 binding, this suggests that, in addition to putatively regulating Rab33 GTP binding at the Golgi membrane, these RabGAPs may in part determine the Golgi membrane local-
The visualization of PI4KB, thereby influencing cellular phosphatidylinositol 4-phosphate (PI4P) levels and Golgi morphology. It is also of note that other RNA viruses and intracellular pathogens use TBC domain-containing proteins and their binding partners to manipulate Rab dynamics at the ER-Golgi interface for their replication, including hepatitis C virus, Legionella and Shigella species, and pathogenic Escherichia coli (21–24).

Given that both TBC1D22A/B and PI4KB share very similar binding sequences and that no ACBD3 mutant could be found that discriminates between these two partners, it remains an open question whether direct regulation of either of these proteins produces modified binding to ACBD3. Mutation of the main phosphorylation site on ACBD3 (SS344) to alanine or glutamic acid had no differential effect on TBC1D22A/B versus PI4KB binding. Conversion of the identified phosphorylation sites on TBC1D22B and PI4KB to glutamic acid also did not disrupt interaction with ACBD3. TBC1D22A/B and PI4KB are organized in similar fashions, with an N-terminal ACBD3 binding domain, a 14-3-3 binding phosphorylation site in the middle, and a C-terminal enzymatic domain. Because 14-3-3 does not bind to phosphomimetic mutations, we were unable to test the effect of 14-3-3 binding on...
interaction with ACBD3 without phosphorylation (15). At present, in the absence of three-dimensional structure information for each of these proteins, it is not clear what regulates the binding of TBC1D22A/B versus PI4KB to the glutamine-rich region of ACBD3.

The picornaviral 3A proteins may inform the cellular biology that regulates whether TBC1D22A/B or PI4KB is bound to ACBD3. An open question in picornavirus biology is why enteroviruses retain a specific GBF1 recruitment domain and are sensitive to brefeldin A while kobuviruses do not bind GBF1 and are insensitive to brefeldin A. Given that multiple picornaviruses rely on ACBD3 and PI4KB, direct competition by TBC1D22A/B suggests that viruses have evolved a mechanism to subvert cellular regulation of these two proteins. Our data show that the kobuviral 3A proteins appear to abrogate the binding of TBC1D22A/B to ACBD3, thus favoring the formation of a stable 3A-ACBD3-PI4KB complex that remains even after 25 to 30 min of washing. This stable complex may obviate the need for GBF1 recruitment and activity and allow kobuviruses to directly influence PI4KB localization and activity with respect to viral replication in a manner similar to, yet distinct from, that of enterovirus 3A. It is also remarkable that a protein that binds entirely to the GOLD domain can manipulate the binding of proteins in other domains of ACBD3, suggesting cross talk between ACBD3’s various domains. Although we have mapped the critical binding region of enterovirus 3A (V45A and H57Y) (25), how these enteroviruses may reflect convergent evolutionary strategies to manipulate this key lipid regulator. The mutants obtained in this study will help illuminate whether different PI4KB mutants can rescue enterovirus replication after depletion of native PI4KB. However, it is of note that enteroviruses selected for resistance to enviroxime family PI4KB inhibitors contain mutations in the ACBD3 binding region of 3A (V45A and H57Y) (25). How these mutants affect ACBD3 binding by 3A and whether PI4KB can compete TBC1D22A/B (see Table S7 in reference 5).

Future experiments with the PI4KB mutants isolated in this study will help illuminate whether different PI4KB mutants can rescue enterovirus replication after depletion of native PI4KB. However, it is of note that enteroviruses selected for resistance to enviroxime family PI4KB inhibitors contain mutations in the ACBD3 binding region of 3A (V45A and H57Y) (25). How these mutants affect ACBD3 binding by 3A and whether PI4KB can compete TBC1D22A/B of ACBD3 are important outstanding questions.

In conclusion, multiple picornaviruses coopt the same central ACBD3-PI4KB axis for replication but utilize different cellular mechanisms to subvert GBF1. The mutants obtained in this study will be useful tools to test whether PI4KB localization to ACBD3 is required for picornavirus replication; to test the effect
of ACBD3, TBC1D22A/B, and GBF1 on P14P levels in the cell; and to discover regulatory mechanisms that govern the ACBD3 inter-actome.

MATERIALS AND METHODS

Cells, plasmids, and cloning. 293T cells were maintained in Dulbecco modified Eagle medium (DMEM)-H21 medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. All genes for transient transfections were cloned into a modified pCDBN4-T4 vector containing an N-terminal or C-terminal 2× Strep II tag as described previously (Table S1 in the supplemental material) (26). Accession numbers used for genes were as follows: ACBD3, NM_002735; TBC1D22A, NM_014346; TBC1D22B, NM_017772; Rab11B. The human CI0orf76 gene was synthesized commercially (BioBasic Inc., Canada).

Transient transfections were performed in a 15-cm plate of 293T cells at 50 to 60% confluence using 10 μg of total plasmid and a 3:1 ratio of TransIT-LT1 transfection reagent to plasmid (Mirus Bio). Protein lysates were prepared in 0.5% NP-40 in a background buffer of either 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA or 50 mM HEPE-S-KOH (pH 6.8), 150 mM potassium acetate (KOAc), 2 mM magnesium acetate (MgOAc), 1 mM CaCl₂, 15% glycerol, 1× Roche EDTA-free protease inhibitor cocktail. Affinity purifications and Western blotting assays were otherwise performed as described previously (5, 26).

For the ACBD3 binding competition assay, transient transfections of ACBD3-Strep, TBC1D22A-Flag, and P4K8-V5 in individual 15-cm plates of 293T cells were performed as described above. Lysates from each plate were prepared in 2 ml of EDTA-containing buffer. P4K8-V5 and ACBD3-Strep lysate were premixed in a 15-ml Falcon tube for 4 h at 4°C. Plates were prepared in 2 ml of EDTA-containing buffer. PI4KB-V5 and plates of 293T cells were performed as described above. Lysates from each ACBD3-Strep, TBC1D22A-Flag, and PI4KB-V5 in individual 15-cm plates of 293T cells were performed as described above. Lysates from each plate were prepared in 2 ml of EDTA-containing buffer. P4K8-V5 and ACBD3-Strep lysate were premixed in a 15-ml Falcon tube for 4 h at 4°C. The lysate was then divided into 7 aliquots of 500 μl each, and 0, 3, 10, 30, 100, 300, and 1,000 μl of lysate from the TBC1D22A-Flag-transfected plate or an untransfected plate was added to the aliquots along with 35 μl of StrepTactin resin (IBA Life Sciences) and incubated overnight at 4°C. ACBD3-Strep was affinity purified, and copurified TBC1D22A-Flag and P4K8-V5 were detected by Western staining using anti-Flag and anti-V5 antibodies (Sigma). For the 3A-TBC1D22A cotransfection experiments (see Fig. S8 in the supplemental material), 2.5 μg of TBC1D22A-Flag and 7.5 μg of picornavirus 3A-Flag were cotransfected using Muris TransIT-LT1 transfection reagent as described above.

Mammalian two-hybrid screening. Interaction mapping was performed by mammalian two-hybrid screening using the Checkmate system (Qiagen). Two micrograms of total RNA from HEK293T cells was reverse transcribed using OligodT12-18, and qRT-PCR was performed using the 480 DNA SYBR Green I master mix (Roche) on a LightCycler (Roche). Primers used were the TBC1D22A-CDS4 and hRPL19 set at a melting temperature (T_m) of 50°C and with an extension of 1 min at 72°C (see Table S1 in the supplemental material).

Protein identification by mass spectrometry. Protein identification from affinity-purified samples was performed using peptide sequencing by mass spectrometry as previously reported (5). Affinity-purified samples were reduced and alkylated with dithiothreitol (DTT) and iodoacetamide, respectively, and then subjected to trypsin digestion either in solution or in excised SDS-PAGE gel bands. Peptide sequencing was performed using an LTQ-FT, an LTQ-Orbitrap XL, or an LTQ-Velos (Thermo) mass spectrometer, each equipped with 10,000-py system nanoACUITY (Waters) ultra-high-performance liquid chromatography (UPLC) instruments for reversed-phase C18 chromatography and using the same data acquisition and processing methods as those previously reported (5).

Database searches were performed against the Homo sapiens plus Virus subset of the NCBI RefSeq database (14 January 2012), to which were added virus clone sequences missing from the public database, totaling 131,457 entries. This database was concatenated with a fully randomized set of 131,457 entries for estimation of the false discovery rate (27). Data were searched with a parent mass tolerance of 20 ppm and fragment mass tolerances of 0.6 Da.

Using peptide counts as an approximation of protein abundance, Z scores were calculated to represent prey-bait specificity as reported previously (5). Z scores for proteins interacting with individual virus or human bait proteins were calculated using a minimum of five replicate experiments together with a background model of 550 control, nonhuman bait data sets. These nonhuman data sets were compiled from the genes from 12 different picornaviruses, excluding the 3A gene itself (VP0/VP2, VP4, VP1, VP3, L, 2A, 2B, 2C, 3C, and 3D), which serve as an unbiased set of proteins that should be orthogonal to the human protein interactions in this network. The nonspecific interacting proteins most commonly identified in this background set, analogous to “frequent fliers” reported as common contaminants in Flag-AP-MS experiments, are reported in Table S2 in the supplemental material (28).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00098-13/-/DCSupplemental.

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