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GBF1, a Guanine Nucleotide Exchange Factor for Arf, Is Crucial for Coxsackievirus B3 RNA Replication▽

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The replication of enteroviruses is sensitive to brefeldin A (BFA), an inhibitor of endoplasmic reticulum-to-Golgi network transport that blocks activation of guanine exchange factors (GEFs) of the Arf GTPases. Mammalian cells contain three BFA-sensitive Arf GEFs: GBF1, BIG1, and BIG2. Here, we show that coxsackievirus B3 (CVB3) RNA replication is insensitive to BFA in MDCK cells, which contain a BFA-resistant GBF1 due to mutation M832L. Further evidence for a critical role of GBF1 stems from the observations that viral RNA replication is inhibited upon knockdown of GBF1 by RNA interference and that replication in the presence of BFA is rescued upon overexpression of active, but not inactive, GBF1. Overexpression of Arf proteins or Rab1B, a GTPase that induces GBF1 recruitment to membranes, failed to rescue RNA replication in the presence of BFA. Additionally, the importance of the interaction between enterovirus protein 3A and GBF1 for viral RNA replication was investigated. For this, the rescue from BFA inhibition of wild-type (wt) replicons and that of mutant replicons of both CVB3 and poliovirus (PV) carrying a 3A protein that is impaired in binding GBF1 were compared. The BFA-resistant GBF1-M832L protein efficiently rescued RNA replication of both wt and mutant CVB3 and PV replicons in the presence of BFA. However, another BFA-resistant GBF1 protein, GBF1-A795E, also efficiently rescued RNA replication of the wt replicons, but not that of mutant replicons, in the presence of BFA. In conclusion, this study identifies a critical role for GBF1 in CVB3 RNA replication, but the importance of the 3A-GBF1 interaction requires further study.

Enteroviruses are small, nonenveloped, positive-stranded RNA viruses that include many important pathogens, such as poliovirus (PV), coxsackievirus, echovirus, and human rhinovirus. Following virus entry and uncoating, the 7.5-kb enteroviral RNA genome is directly translated into a large polyprotein. This polyprotein is proteolytically processed by the virus-encoded proteases $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$ into the structural P1 region proteins and the nonstructural P2 and P3 region proteins that are involved in viral RNA replication.

All RNA viruses with a positive-stranded genome induce the remodeling of cellular membranes to create a scaffold for genomic RNA replication. The organelle origin and morphology of these membranous replication sites, however, appear to vary for different viruses. Enteroviruses replicate their RNA genomes in nucleoprotein complexes that are associated with small vesicular membrane structures (6). The enteroviral proteins 2B, 2C, and 3A have been implicated in vesicle formation (4, 6, 27), but the mechanism and pathway of membrane reorganization are poorly understood. There are strong indications that these vesicular membranous structures, which are referred to here as "vesicles," are derived from the early secretory pathway. Vesicles produced in PV-infected cells may form at the endoplasmic reticulum (ER) by the cellular COP-II budding machinery and may therefore share components with the membranous vesicles mediating ER-to-Golgi network transport (26). Further support for the involvement of the secretory pathway stems from the observation that brefeldin A (BFA), a well-known inhibitor of ER-to-Golgi network transport, completely inhibits enteroviral RNA replication (17, 20). In addition, the autophagocytic pathway appears to contribute to the formation of the membrane vesicles, many of which exhibit a double-membrane morphology characteristic of autophagosomes (18, 27). The utilization of individual components or reactions from different membrane metabolic pathways, rather than subversion of an entire pathway in toto, may represent a common strategy for building viral replication machinery.

BFA inhibits activation of the small monomeric GTPase ADP ribosylation factor 1 (Arf1), a major regulator of intracellular protein transport (2). Arf1 cycles between an inactive, GDP-bound, cytosolic state and an active, GTP-bound, membrane-associated state, and this cycling is catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (13). BFA blocks the activities of the large GEFs GBF1, BIG1, and BIG2 by stabilizing an intermediate, abortive complex with inactive Arf1 (23), thus efficiently preventing activation of Arf1 and eventually formation of transport intermediates.

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Not only the fact that BFA blocks enteroviral replication suggests a role for Arf1 and/or its large GEFs in this process; recently, it was shown that Arf1 accumulates on membranes during PV infection (3). Arf1 translocation to membranes can be induced independently by enterovirus protein 3A or 3CD in vitro (5), but the underlying mechanisms seem to differ; the 3A protein specifically triggers the recruitment of GBF1 to membranes, most likely through a direct interaction with this GEF (32, 33), whereas 3CD recruits BIG1 and BIG2 to membranes (3). Here, we report the involvement of Arf1 and its large BFA-sensitive GEFs in coxsackievirus B3 (CVB3) replication.

MATERIALS AND METHODS

Cells and reagents. Buffalo green monkey (BGM) kidney cells and HeLa cells were grown in minimal essential medium (Gibco) supplemented with 10% fetal bovine serum (FBS). Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's minimum essential medium (Gibco) supplemented with 10% FBS. The cells were grown at 37°C in a 5% CO_2 incubator. BFA was purchased from Sigma-Aldrich and was used, unless otherwise stated, at a final concentration of 2 μg/ml.

Viruses and replicons. The CVB3 used in this study was derived from the p53CB3/T7 plasmid, which contains the cDNA of CVB3 strain Nancy driven by a T7 RNA polymerase promoter (34). CVB3 expressing either *Renilla* luciferase (RLuc-CVB3) or enhanced green fluorescent protein (EGFP-CVB3) were obtained by placing the *Renilla* luciferase or EGFP coding sequences, followed by a 3CD cleavage site, between the 5′ untranslated region and the P1 coding region. Virus yields were determined by end-point titration according to the method of Reed and Muench and expressed as 50% tissue culture infective doses ($TCID_{50}$) (24). The CVB3 replicons pRib-CB3-LUC and pRib-CB3-RLUC contain a CVB3 cDNA in which the P1 capsid coding region has been replaced by the firefly luciferase gene or the *Renilla* luciferase gene, respectively. The cDNA was placed behind a hammerhead ribozyme coding sequence to remove 5′-end extra nucleotides. The encephalomyocarditis virus (EMCV) replicon pE-Luc (1) and the PV replicons pXpA-RenR and pXpA-RenR 3A-2 (4) have been described previously.

Plasmids. Plasmids pEYFP-GBF1 wild type (wt), M832L, E794K, ΔSec7 (22), A795E (4), and pRab1b-EGFP wt and the Q67L mutant (21) have been previously described. Enhanced yellow fluorescent protein (EYFP)-tagged versions of the different Arf isoforms have been described previously (14). Q71L versions of these EYFP-tagged Arf isoforms were constructed by site-directed mutagenesis. Plasmid pCMV-Gluc, which expresses secreted *Gaussia* luciferase, was purchased from New England Biolabs.

Virus infection and BFA rescue experiments. Confluent monolayers of BGM or HeLa cells were infected with virus for 30 min at 37°C at a multiplicity of infection (MOI) of 10 $TCID_{50}$ per cell. The cells were then washed twice with phosphate-buffered saline and cultured in minimal essential medium supplemented with 10% FBS, with or without BFA, at 37°C for the indicated periods of time. At the indicated times, the cells were disrupted by three cycles of freezing and thawing, and virus titers were determined by end-point titration. For the rescue experiments, BGM or HeLa cells were grown in 24-well plates to subconfluence and transfected with 200 ng plasmid DNA using GeneJuice Transfection reagent (Novagen) according to the manufacturer's instructions. At 16 h posttransfection, the cells were infected with virus at an MOI of 10 as described above.

Replicon assays. Replicons were linearized, purified, and transcribed in vitro by T7 RNA polymerase, and transcript RNAs were transfected into cells as described previously (4, 28). After transfection, the cells were cultured in medium with or without BFA at 37°C. At the indicated times posttransfection, the cells were lysed and luciferase activity was assayed as described previously (4, 28).

Immunofluorescence. MDCK cells grown to subconfluence on coverslips in 24-well plates were treated with BFA for 2 h at 37°C and stained with polyclonal anti-COP-I (against α- and γ-COP) antiserum (diluted 1:200; from K. Frey and F. Wieland, Biochemie-Zentrum, Heidelberg, Germany) or monoclonal anti-adaptin (AP-1) antiserum (diluted 1:100; Sigma). Alexa Fluor 594-conjugated goat anti-rabbit immunoglobulin G and Alexa Fluor 488-conjugated anti-mouse immunoglobulin G were obtained from Molecular Probes. Pictures were taken with a Leica TCS NT microscope (Leica Lasertechnik GmbH, Heidelberg, Germany).

siRNA treatments. HeLa Kyoto cells were plated at 40% to 50% confluence in 24-well plates, with or without coverslips, and transfected with 50 pmol of small interfering RNA (siRNA) using Oligofectamine (Invitrogen) according to the manufacturer's protocol. The siRNAs directed against GBF1 and Arf were from Qiagen (identifier, SI00425411) and Ambion (identifier, 10237), respectively. At 40 h posttransfection, the cells were infected with either a *Renilla* luciferase (RLuc-CVB3)- or EGFP (EGFP-CVB3)-expressing virus at an MOI of 5 $TCID_{50}$ per cell. At 7 h postinfection, the cells were either fixed and Hoechst counterstained before the fluorescent cells were counted, or the cells were lysed in *Renilla* lysis buffer and the luciferase activity was assayed as described previously (28).

Immunoblotting. Protein samples were run on a NuPage 4 to 12% *N*,*N*-methylenebisacrylamide–Tris gradient gel (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad). EYFP fusion proteins were stained with the anti-EGFP polyclonal antiserum (dilution 1:10,000) (10) or anti-EGFP monoclonal antibodies (Becton Dickinson).

Secretion rescue assay. HeLa cells were plated at 40,000 per well in a 96-well plate and transfected with pGEM (control), pEYFP-GBF1 M832L, or pEYFP-GBF1 A795E and pCMV-Gluc with Lipofectamine LTX (Invitrogen). The next day, the cells were washed and incubated with medium containing 1 μg/ml BFA for 5 h. A portion of the medium was tested for secreted luciferase activity with the Gaussia Luciferase assay kit (New England Biolabs).

BFA toxicity assay. HeLa cells were plated at 20,000 per well in a 96-well plate and transfected with pGEM, pEYFP-GBF1 M832L, or pEYFP-GBF1 with Lipofectamine LTX. The next day, the cells were incubated in medium containing the indicated amount of BFA. The medium was changed at regular intervals to ensure constant pressure of the inhibitor. Cell viability was determined at 2 days posttransfection using the Cell Titer-Glo luminescence cell viability assay kit (Promega).

RESULTS

GBF1 activity, but not BIG1 or BIG2 activity, is required for CVB3 RNA replication in MDCK cells. BFA inhibits the three large Arf GEFs GBF1, BIG1, and BIG2 (Fig. 1A) by binding to and stabilizing transient complexes between the GEF and an inactive, GDP-bound Arf. To determine the relative contributions of the individual GEFs to viral replication, we investigated CVB3 RNA replication in MDCK cells. The sequence of the dog genome indicates a naturally occurring mutation in the Sec7 domain of GBF1 (M832L) that is predicted to render it insensitive to BFA (22), whereas BIG1 and BIG2 in these cells are predicted to be BFA sensitive (19) (Fig. 1A). Sequence analysis of the Sec7 domain of the MDCK cell line that was used in our study confirmed the occurrence of the M832L mutation (data not shown). The resistance of GBF1 in these cells to BFA was demonstrated by immunofluorescence studies. GBF1 mainly resides in the *cis*-Golgi, where it activates Arf1 to recruit COP-I, whereas BIG1 and BIG2 mainly reside in the *trans*-Golgi network (TGN) and endosomes, where they activate Arf1 to recruit AP-1. Due to this compartmentalization, BFA treatment of MDCK cells caused AP-1 to dissociate from TGN and endosomal membranes, whereas COP-I remained associated with *cis*-Golgi membranes (Fig. 1B) (35), consistent with a resistance of GBF1, but not BIG1 and BIG2, to BFA.

CVB3 cannot infect MDCK cells (data not shown), most likely due to absence of the receptor on the cell surface. Therefore, we transfected these cells with a subgenomic replicon of CVB3 containing the firefly luciferase gene in place of the P1 coding region (pRib-CB3-LUC). The amount of luciferase activity produced by this replicon is a measure of viral-RNA replication. MDCK cells transfected with RNA transcripts of the CVB3 replicon were incubated for the indicated periods of time in the presence or absence of BFA or guanidine-HCl, a well-known inhibitor of enterovirus replication (25). Analysis of the luciferase activity showed that CVB3 replicates effi-

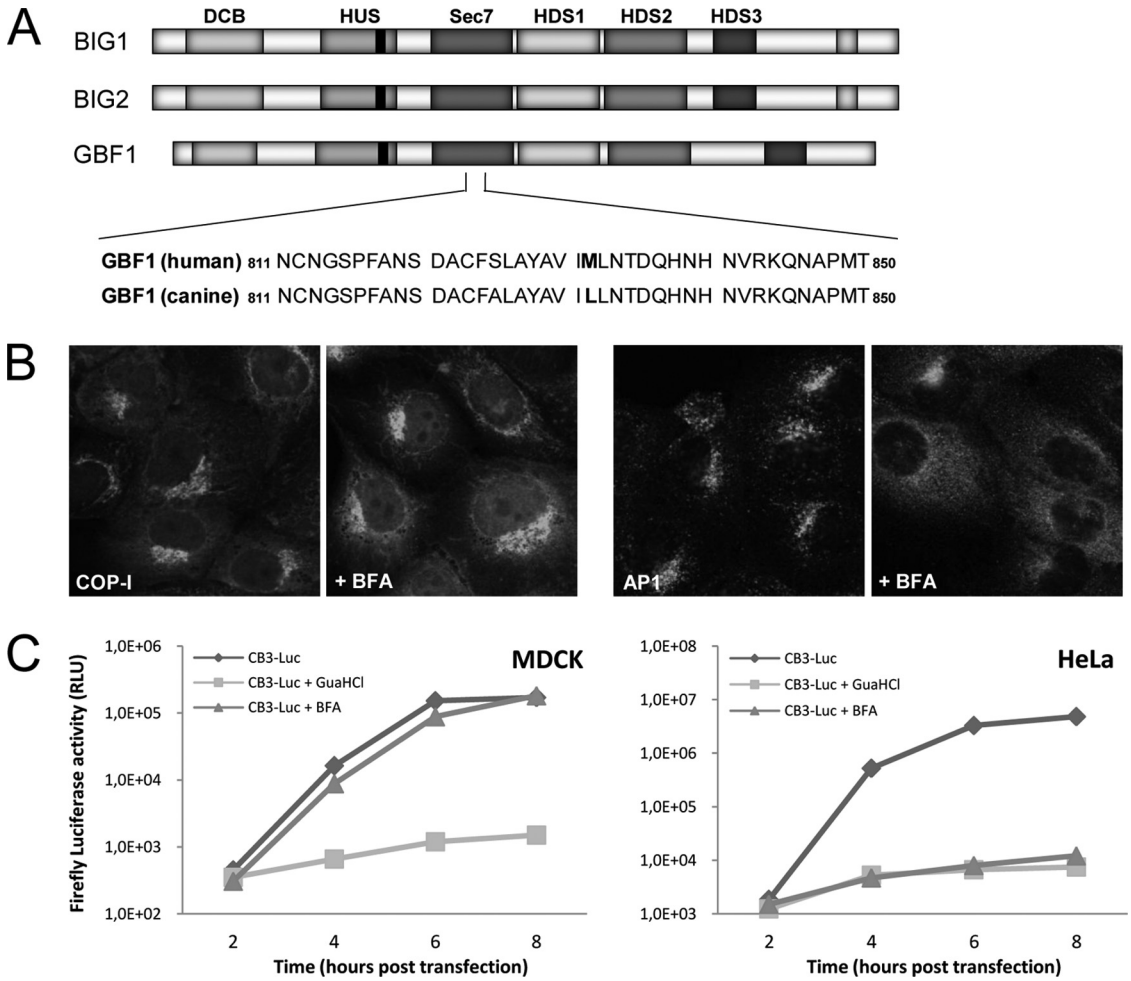

FIG. 1. BFA does not affect CVB3 RNA replication in MDCK cells, which contain a BFA-resistant GBF1 protein. (A) Schematic representation of the three BFA-sensitive Arf GEFs. The BFA-resistant mutation in the Sec7 domain of GBF1 in MDCK cells is indicated in boldface. (B) Control MDCK cells and cells treated with 2 μg/ml BFA were stained with antibodies against COP-I or AP1. Note that BFA has no effect on the association of COP-I with Golgi membranes but causes the dissociation of AP1 from the TGN and endosomes in MDCK cells. (C) MDCK cells and HeLa cells were transfected with in vitro RNA transcripts derived from a cDNA encoding a CB3 replicon containing the firefly luciferase gene in place of the P1 capsid coding region. After transfection, the cells were supplied with medium containing BFA or guanidine-HCl, a well known inhibitor of enterovirus RNA replication. Luciferase production was measured at the indicated time points.

ciently in the presence of BFA in MDCK cells, whereas replication was completely blocked by BFA in HeLa cells (Fig. 1C). Since GBF1 is the only GEF resistant to BFA in MDCK cells, this indicates a role for GBF1, but not for BIG1 and BIG2, in CVB3 replication.

Knockdown of GBF1, but not Arf1, reduces CVB3 replication in HeLa cells. Thus far, the potential roles of host cell proteins in CVB3 replication had been studied in the presence of BFA. To further explore the possible requirement for GBF1 and Arf1 in CVB3 replication, HeLa cells were depleted of GBF1 or Arf1 by treatment with specific siRNAs. Analysis of the efficiency of knockdown with an antibody against GBF1 showed that siRNA treatment reduced the expression of the protein in ~80% to 90% of the cells (Fig. 2A). Due to the lack of commercial antibody that specifically recognizes Arf1, but not the other Arf isoforms, another procedure was employed to demonstrate the efficiency of the Arf1 siRNA treatment. For this, cells were transfected with plasmids expressing an EYFP fusion of either GBF1 or Arf1, together with siRNAs against GBF1 or Arf1. The fluorescence of EYFP-Arf1 and EYFP-GBF1 was lost after cotransfection with the corresponding siRNA, showing that the knockdown was efficient (Fig. 2B). No reduction of EYFP-Arf1 or EYFP-GBF1 fluorescence was observed upon cotransfection of a scrambled control siRNA.

To examine the effects of the knockdowns on CVB3 RNA replication, we made use of recombinant viruses expressing either EGFP (EGFP-CVB3) or *Renilla* luciferase (RLuc-CVB3). These foreign sequences were inserted between the 5′ untranslated region and the capsid-coding region and were followed by a $3CD^{pro}$ cleavage site to allow proteolytic processing of the foreign proteins from the viral polyprotein. These viruses showed efficient RNA replication, but virus production was impaired relative to wt virus, due to inefficient liberation of the foreign proteins from the viral polyprotein and/or inefficient packaging of the viral RNAs containing the insert. The amount of fluorescence or luciferase activity is a

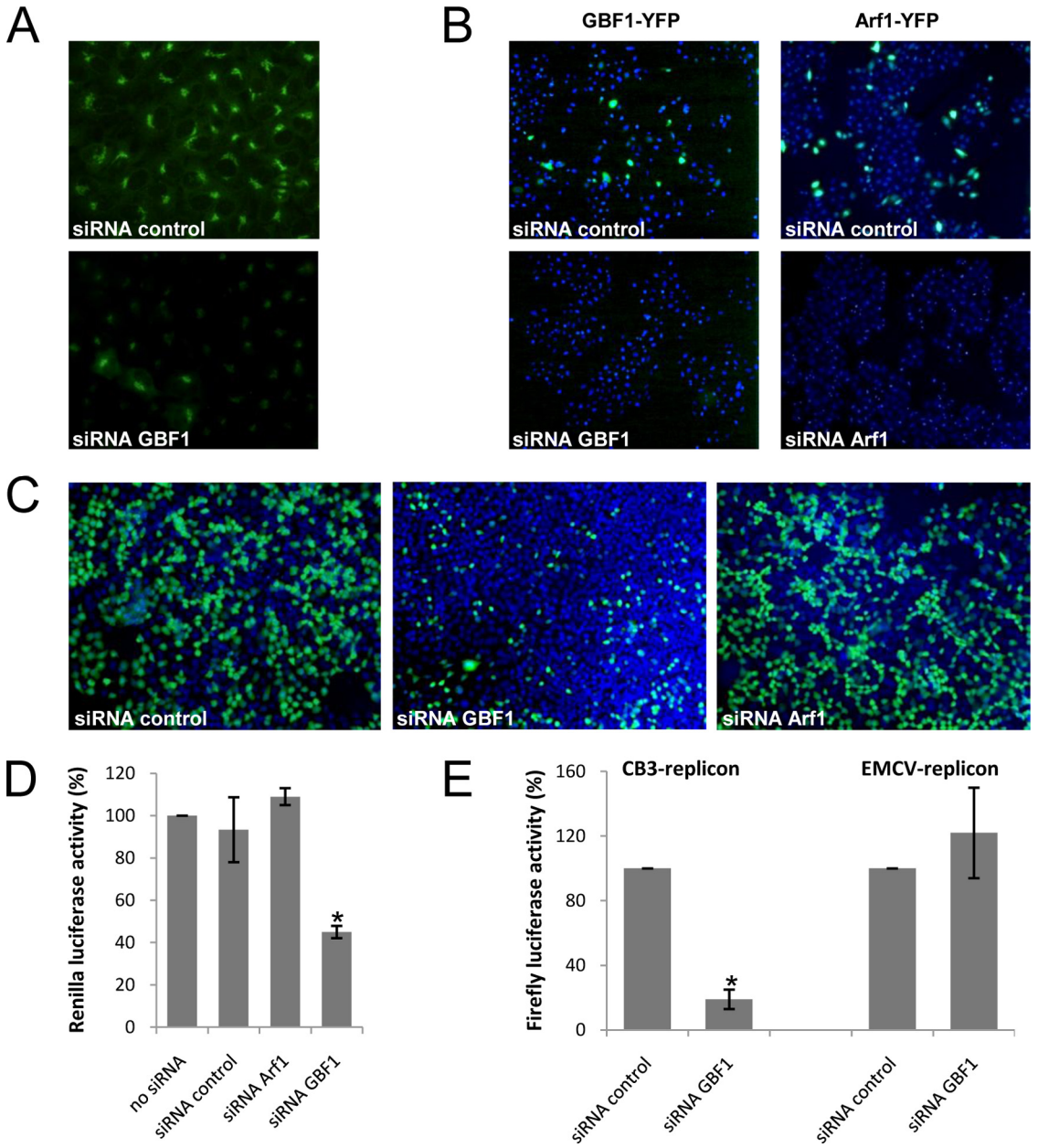

FIG. 2. siRNA-mediated knockdown of GBF1, but not of Arf1, inhibits CVB3 RNA replication in HeLa cells. (A) Cells were treated with siRNA against GBF1 for 40 h and then stained with an antibody against GBF1. The siRNA treatment inhibited GBF1 expression in about 80 to 90% of the cells. (B) Cells were cotransfected with a plasmid encoding EYFP-GBF1 and siRNA against GBF1 (left) or cotransfected with a plasmid encoding Arf1-EYFP and siRNA against Arf1. Fluorescence was monitored at 2 days posttransfection. (C) Cells were treated with siRNAs directed against GBF1 or Arf1 or a scrambled siRNA (control). At 40 h posttransfection, the cells were infected with GFP-expressing CVB3. GFP fluorescence, which is indicative of replication, was monitored at 7 h postinfection. (D) Similar to panel C, but the cells were infected with a *Renilla* luciferase-expressing CVB3. Luciferase production was measured at 7 h postinfection and is expressed as a percentage of that observed with the no-siRNA control (which was set at 100%). (E) Cells were treated with siRNAs against GBF1 or a scrambled siRNA and then transfected with in vitro transcripts from firefly luciferase-expressing replicons of CVB3 or EMCV. The amount of luciferase produced at 8 h posttransfection was measured and is expressed as a percentage of that observed with the siRNA control (which was set at 100%).

good measure of viral RNA replication. HeLa cells were treated with siRNA for 40 h, infected with either EGFP-CVB3 or RLuc-CVB3, and analyzed at 7 h postinfection. Treatment of cells with GBF1 siRNA reduced the relative number of fluorescent cells (Fig. 2C), as well as the amount of luciferase produced (Fig. 2D). No inhibition of CVB3 replication was observed upon Arf1 knockdown; the number of fluorescent cells (Fig. 2C) and the levels of luciferase in cells treated with Arf1 siRNA (Fig. 2D) were similar to those in control siRNA-treated cells. To exclude the possibility that the inhibitory effect of GBF1 depletion on CVB3 replication is due to a general toxic effect on the cells, RNA replication levels of

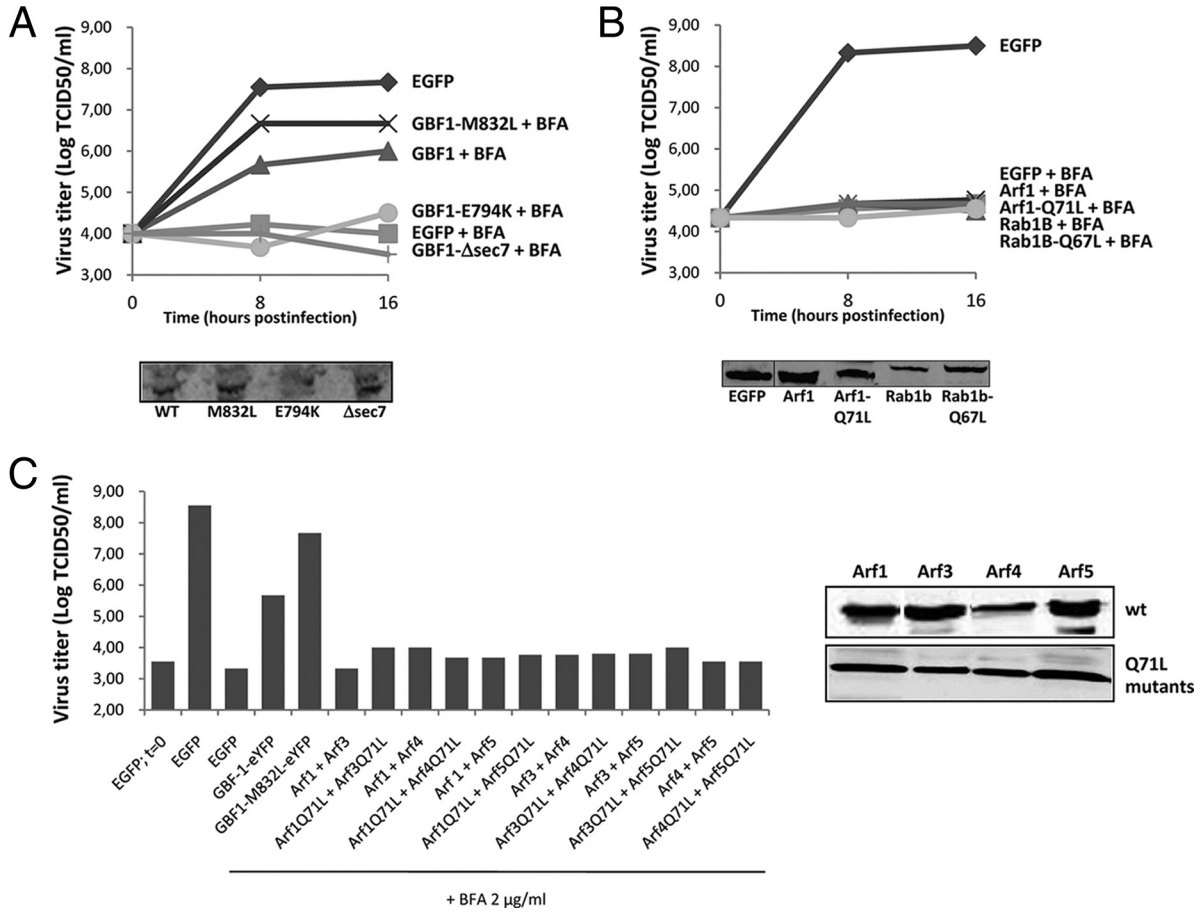

FIG. 3. GBF1, but not Arfs or Rab1B, rescues CVB3 replication in the presence of BFA. BGM cells were transfected with the indicated GBF1 expression constructs (A), with the indicated Arf1 or Rab1b expression constructs (B), or with combinations of different Arf isoforms (C). All of the expressed proteins were GFP tagged. The next day, the cells were infected with CVB3 at an MOI of 10 and incubated at 37°C in medium with or without BFA. At 8 h (C) or at 8 and 16 h (A and B) postinfection, the cells were lysed by three cycles of freezing and thawing, and the amount of virus was determined by end-point titration. Western blot analysis using an anti-EGFP serum confirmed the expression of the indicated proteins.

luciferase-expressing subgenomic replicons of CVB3 and EMCV, a picornavirus whose replication is insensitive to BFA (16), were compared. Figure 2E shows that the EMCV replicon (pE-Luc) replicated efficiently in GBF1 siRNA-treated cells, whereas RNA replication of the CVB3 replicon (pRibCB3-LUC) was strongly reduced. The use of a replicon to measure the effects on RNA synthesis additionally eliminated virus adsorption, entry, and assembly processes as being responsible for the virus growth inhibition by GBF1 knockdown. Collectively, these data show that GBF1 activity is crucial for RNA replication of CVB3.

Overexpression of GBF1, but not Arf1 or Rab1B, rescues CVB3 replication in the presence of BFA. Overexpression of GBF1 has been shown to (partially) rescue PV replication in the presence of BFA (3). To investigate whether overexpression of GBF1 could also rescue CVB3 replication in the presence of BFA, BGM cells were transfected with plasmid expressing EYFP-GBF1 or, as a control, EGFP prior to infection in the presence or absence of BFA. In the absence of BFA, CVB3 replication levels in cells transfected with EGFP and EYFP-GBF1 were indistinguishable (data not shown). Figure 3A shows that overexpression of GBF1, but not EGFP, partially rescued the effect of BFA on CVB3 replication (for reasons of clarity, in this figure and subsequent figures, CVB3 replication in cultures transfected with only one of the constructs in the absence of BFA is shown). Overexpression of a GBF1 protein containing mutation M832L (i.e., the mutation that rendered MDCK cells resistant to BFA) resulted in a much stronger rescue of the effects of BFA, as indicated by a 10-fold increase in the virus titer. Overexpression of inactive GBF1 proteins, either lacking the catalytic Sec7 domain (GBF1ΔSec7) or containing mutation E794K in this domain (15), failed to rescue CVB3 replication in the presence of BFA (Fig. 3A). Overexpression of BIG2 also failed to rescue CVB3 replication in the presence of BFA (data not shown; BIG1 was not tested since heterologous expression of this protein in mammalian cells has not been successful). Similar results were obtained in HeLa cells. Together, these data suggest that active GBF1 is required to support CVB3 replication.

We also tested whether overexpression of Arf1 could rescue CVB3 replication in the presence of BFA. Neither wt Arf1 nor Arf1-Q71L, a constitutive active mutant of Arf1, could rescue CVB3 replication in the presence of BFA (Fig. 3B). Similarly,

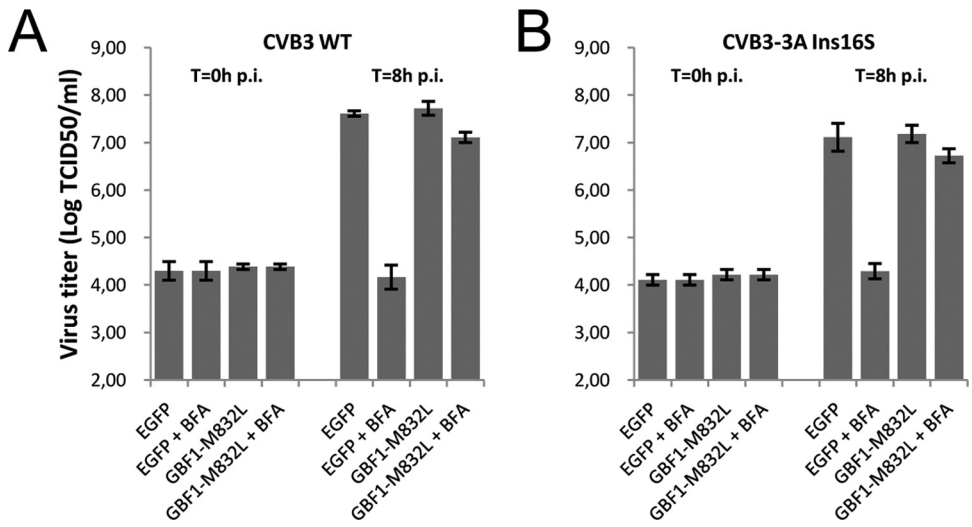

FIG. 4. GBF1-M832L efficiently rescues replication of CVB3 carrying a mutant 3A protein that is impaired in binding GBF1 in the presence of BFA. BGM cells were transfected with EGFP (control) or EYFP-GBF1 M832L. The next day, the cells were infected with either wt CVB3 (A) or CVB3-3A-ins[16]S (B) at an MOI of 10 and incubated at 37°C in medium with or without BFA. Virus titers were determined at 0 h and 8 h postinfection (p.i.). The error bars indicate standard deviations.

no rescue was observed upon overexpression of wt Arf3, Arf4, or Arf5 or the dominant active Q71L mutants of these Arf proteins (data not shown). Recently, evidence was presented that different Arf isoforms act in pairs at distinct sites in the secretory pathway (30). Therefore, we also tested whether these different combinations of Arf isoforms (both the wt and the constitutive active Q71L mutants) could rescue replication. The results showed that neither of the tested combinations of Arf isoforms could rescue CVB3 replication in the presence of BFA (Fig. 3C).

Rab proteins, small GTPases, regulate membrane transport between various compartments within eukaryotic cells. Rab proteins regulate the formation, tethering, docking, and fusion of vesicular membranes by recruiting and interacting with downstream effector proteins. Rab1b interacts with the N terminus of GBF1 and induces GBF1 recruitment at ER exit sites and Golgi membranes (21). However, overexpression of neither wt Rab1B nor the constitutive active mutant Rab1B-Q67L could rescue CVB3 replication in the presence of BFA (Fig. 3B).

GBF1-M832L efficiently rescues replication of CVB3 carrying a mutant 3A protein that is impaired in binding GBF1 in the presence of BFA. The CVB3 3A protein has been shown to interact with the N terminus of GBF1 (i.e., the region upstream of the catalytic Sec7 domain) (33). To investigate the importance of this interaction for CVB3 replication, we analyzed the GBF1-mediated rescue of CVB3-3A-ins[16]S. This virus, which shows delayed replication kinetics compared to wt virus but which reaches a comparable titer after 8 h (31), contains a serine insertion at amino acid position 16 in the 3A protein. This mutant 3A protein showed a severely reduced interaction with GBF1 (31). BGM cells transiently expressing GBF1-M832L were infected with either CVB3 wt or the CVB3-3A-ins[16]S mutant in the presence or absence of BFA, and virus production was measured at 8 h postinfection. Figure 4 shows that replication of both wt CVB3 and the CVB3-3A-ins[16]S mutant in the presence of BFA was efficiently rescued by GBF1-M832L overexpression. Similar results were obtained in HeLa cells (data not shown).

Differential rescue abilities of BFA-resistant GBF1 mutants M832L and A795E. We have previously demonstrated that GBF1 plays an important role in PV replication (4). In this study, another BFA-resistant mutation in GBF1, mutation A795E, was identified. This mutation was identified in a BFA-resistant line of Vero cells. Overexpression of GBF1-A795E rescued RNA replication of a wt PV replicon (pXpA-RenR) in the presence of BFA. This GBF1 mutant, however, only poorly rescued replication of a mutant PV replicon (mutant 3A-2) carrying a serine insertion in the 3A protein at position 15 (which corresponds to the 3A-ins[16]S mutation in CVB3 3A). Thus, a BFA-resistant GBF1 protein (M832L) efficiently rescued CVB3 carrying a 3A mutation with impaired GBF1 binding, whereas another BFA-resistant GBF1 protein (A795E) did not efficiently rescue a PV replicon harboring the same 3A mutation.

To elucidate the underlying reason for this apparent contradiction, we compared the RNA replication levels of both wt and mutant *Renilla* luciferase-expressing CVB3 and PV replicons in the presence or absence of BFA in cells transiently expressing either GBF1-M832L or GBF1-A795E. Figure 5 shows that replications of the wt CVB3 replicon and the mutant CVB3 replicon carrying mutation 3A-ins[16]S in the presence of BFA were equally well rescued by the GBF1-M832L protein, consistent with the results shown in Fig. 4. GBF1-A795E, however, rescued RNA replication of the wt CVB3 replicon to a significantly greater extent than that of the mutant 3A-ins[16]S replicon, which was hardly rescued by this GBF1 mutant. A similar trend was observed with the PV replicons. Replication of both the wt PV replicon and the 3A-2 mutant replicon in the presence of BFA was efficiently rescued by GBF1-M832L, whereas GBF1-A795E showed better rescue of the wt replicon than of the mutant 3A-2 replicon. Thus, the

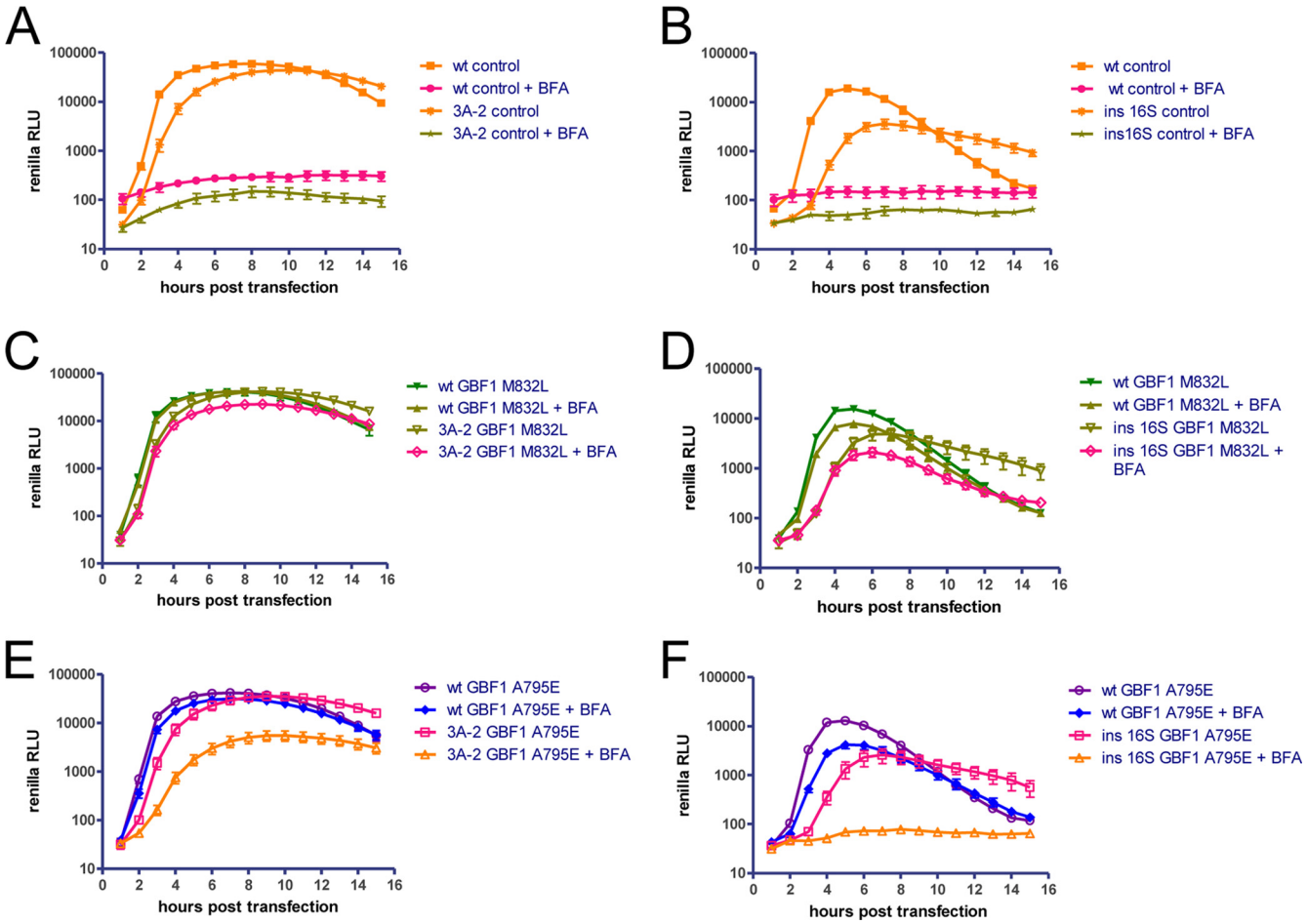

FIG. 5. Differential viral-RNA rescue effects of BFA-resistant GBF1 mutants M832L and A795E in HeLa cells. RNA replication of wt and mutant replicons of PV (A, C, and E) or CVB3 (B, D, and F) in the presence or absence of BFA in nontransfected (control) cells (A and B), in cells transiently transfected with GBF1 M832L (C and D), or in cells transiently transfected with GBF1 A795E. RNA replication was measured by determining the amount of *Renilla* luciferase production.

difference in the observed abilities of BFA-resistant GBF1 proteins to rescue replication of a CVB3 3A mutant (Fig. 4) and a PV 3A mutant (4) can be explained by the use of different BFA-resistant GBF1 mutants.

We previously showed that overexpression of GBF1 could rescue secretory pathway transport, as well as cell viability, in the presence of BFA (4). We compared the abilities of the two GBF1 mutants to rescue protein secretion and cell viability in the presence of BFA. To measure protein secretion, cells were cotransfected with a plasmid expressing *Gaussia* luciferase with a secretion signal and the indicated GBF1 mutants or a control plasmid. The results (Fig. 6A) showed that GBF1-M832L was more effective in rescuing secretion of *Gaussia* luciferase in the presence of BFA than GBF1-A795E. Mutant GBF1-M832L was also more effective than GBF1-A795E in conferring resistance to cell death induced by various concentrations of BFA (Fig. 6B). Western blot analysis of cell extracts showed equal amounts of the two GBF1 mutants, confirming that the observed differences in rescue abilities are unlikely to be due to differences in expression levels.

DISCUSSION

Enteroviruses replicate their RNA genomes at the outer surfaces of rearranged, vesicular membrane structures that accumulate in the cytoplasm of the infected cell. RNA replication is sensitive to BFA, an inhibitor of ER-to-Golgi network transport that inhibits Arf activation by interfering with the activities of large Arf GEFs. Mammalian cells contain three large BFA-sensitive Arf GEFs: GBF1, BIG1, and BIG2 (13). In this study, we presented three lines of evidence that GBF1 plays a critical role in CVB3 RNA replication. First, we showed that RNA replication of CVB3 was insensitive to BFA in MDCK cells, which express a mutant GBF1 protein (mutation M832L) that is resistant to BFA (Fig. 1). Second, knockdown of GBF1 expression by RNA interference inhibited CVB3 RNA replication (Fig. 2). Under the same conditions, no effect on RNA replication of EMCV, a BFA-insensitive picornavirus, was observed, demonstrating the specificity of the approach. Third, overexpression of GBF1, in particular the M832L mutant, rescued the replication of CVB3 in the presence of BFA (Fig. 3). No rescue was observed with inactive mutants of

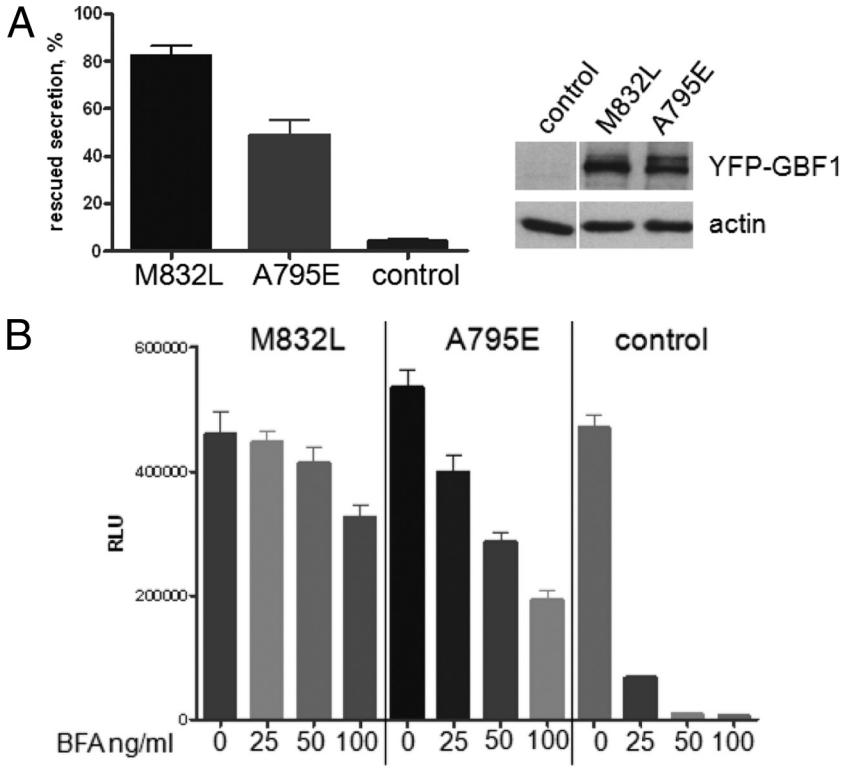

FIG. 6. BFA-resistant GBF1 mutants M832L and A795E differ in their abilities to rescue protein secretion and cell viability in the presence of BFA. (A) HeLa cells were cotransfected with plasmid pCMV-Gluc expressing secreted *Gaussia* luciferase and either a control plasmid or plasmids expressing GBF1 mutant M832L or A795E. The next day, protein secretion in the presence of 1 μg/ml BFA was monitored. The amount of secreted protein in each sample in the absence of BFA was set at 100%. Western blot analysis (right) showed equal expression of the transfected GBF1 mutant proteins. (B) HeLa cells were transfected with a control plasmid or plasmids expressing GBF1 mutant M832L or A795E, and subsequent cell growth in the presence of the indicated amounts of BFA was monitored by a luminescent cell viability assay. The error bars indicate standard deviations.

GBF1. Overexpression of wt or dominant active forms of Arf proteins or Rab1B, a small GTPase that recruits GBF1 to membranes, also failed to rescue CVB3 RNA replication in the presence of BFA. We have previously shown the importance of GBF1 for PV RNA replication (4). Together, these data strongly support a crucial role of GBF1 activity in enterovirus RNA replication. The role of GBF1 in the replication of positive-stranded RNA viruses does not seem to be limited to enteroviruses. Recently, RNA replication of mouse hepatitis virus, a coronavirus, was also shown to rely on GBF1 activity (29).

All three large BFA-sensitive Arf GEFs have been shown to be recruited to membranes by enteroviral nonstructural proteins. GBF1 is recruited to membranes in HeLa cell lysates expressing the 3A protein (3) and has been recognized to interact physically with the 3A protein (33). BIG1 and BIG2 have been shown to be recruited to membranes in HeLa cell lysates expressing the 3CD protein. We observed that CVB3 replication in MDCK cells was insensitive to BFA. In these cells, the activity of GBF1, but not that of BIG1 and BIG2, is insensitive to BFA. This finding suggests that the BFA-sensitive functions of BIG1 and BIG2 are not required for enterovirus RNA replication. A similar conclusion was drawn by Belov et al. (4), who observed efficient replication of PV in a BFA-resistant Vero-derived cell line (BER-40). This cell line contained a BFA-resistant mutation in the Sec7 domain of GBF1 (A795E) but no mutations in the Sec7 domain of BIG1 or BIG2. Together, these data suggest that BFA blocks enterovirus replication by inhibiting the activity of GBF1, but not that of BIG1 and BIG2. Obviously, these data cannot exclude the possibility that BIG1 and/or BIG2 exerts BFA-independent functions that are required for enterovirus RNA replication. However, the observation that knockdown of BIG1 and BIG2 did not affect PV replication in cultured cells (Q. Feng, G. Belov, and E. Ehrenfeld, unpublished results) argues against this possibility.

Previously, we showed that the 3A proteins of both CVB3 and PV interact with GBF1 (32, 33). Whether this interaction is required for viral RNA replication, however, remains to be established. Three observations suggest that this may be the case. First, GBF1 was shown to relocalize to the 3A-containing virus-induced membrane structures at which viral RNA replication takes place in infected cells (3). Second, overexpression of the BFA-resistant GBF1-A795E mutant efficiently rescued RNA replication of wt CVB3 and PV RNA, but not of 3A mutant viruses that are impaired in interacting with GBF1 (Fig. 5) (4). Third, replication of PV RNA carrying a 3A mutation that impaired its interaction with GBF1 (mutation 3A-2) was much more sensitive to BFA than wt PV RNA (5). However, three other observations question the importance of

the 3A-GBF1 interaction for efficient viral RNA replication. We observed no difference in the BFA sensitivities of replication of wt CVB3 RNA and a mutant RNA carrying the corresponding mutation in 3A (ins[15]S) (data not shown). In addition, we found that overexpression of the BFA-resistant mutant GBF1-M832L rescued RNA replication of the 3A mutant viruses of both CVB3 and PV in the presence of BFA, as well as that of the wt viruses (Fig. 4). Finally, RNA replication of human rhinoviruses, which contain either a 3A protein that only weakly interacts with GBF1 (HRV14) or that fails to interact with GBF1 (HRV2) (32), is also completely abolished by BFA (reference 17 and data not shown).

In this study, we observed that the GBF1-M832L mutant was more effective in rescuing protein secretion and cell viability in the presence of BFA than the GBF1-A795E mutant (Fig. 6); thus, the former variant is more resistant to BFA than the latter. This finding may explain why GBF1-M832L was more effective in rescuing RNA replication of the CVB3 and PV 3A mutant viruses in the presence of BFA than the GBF1-A795E mutant (Fig. 5). Although the structure of the GBF1 Sec7 domain has not been resolved, extrapolation of the crystal structure of the Sec7 domain of another GEF, Arno, complexed with Arf and BFA suggests that the GBF1 M832 and A795 residues would be located in close proximity and that both residues participate in van der Waals contacts with BFA. The potential interactions between 3A, GBF1, Arf-GDP, and BFA are not understood at the molecular level. For example, 3A binding to GBF1 may occur with quite different kinetics and affinities than its binding to the GBF1-BFA-Arf complex. Thus, the expected effects of specific mutations in 3A or in GBF1 are very difficult to predict. Therefore, it remains to be established whether the 3A-GBF1 interaction is required to create the appropriate conditions for viral RNA replication or whether, alternatively, GBF1 activity is required for viral RNA replication independent of a functional interaction with 3A. In the latter case, the 3A-GBF1 interaction may be primarily involved in the 3A-mediated inhibition of secretory pathway transport (7, 8, 12, 34), a function that has been implicated in the viral suppression of cytokine secretion and major histocompatibility complex-dependent antigen presentation (9, 11).

Our results showed that knockdown of Arf1 had no effect on CVB3 replication (Fig. 2). This finding may seem surprising given the inhibition of replication by BFA and the observed recruitment of Arf1 to the enterovirus-induced membrane vesicles (5). However, the interpretation of this observation should take into account the results reported by Volpicelli-Daley et al. (30), who performed a comprehensive study of the roles of the different Arf isoforms in membrane traffic. In that study, it was demonstrated that depletion by siRNA of Arf1, Arf3, Arf4, and Arf5 did not affect any step of membrane traffic in HeLa cells. However, every combination of the double knockdowns of these Arf isoforms yielded a distinct pattern of defects in secretory and endocytic traffic, demonstrating clear specificity for Arfs at multiple steps, as well as cooperation of two Arfs at specific sites (e.g., Arf1 and Arf4 were recognized to be required for anterograde transport between the ER and the ERGIC, whereas Arf1 and Arf3 were shown to be important for ERGIC-to-Golgi network transport). Translation of PV RNA in HeLa cell lysates recruits not only Arf1 to membranes, but also the other Arf isoforms (except Arf6, which acts mainly in vesicular processes near the plasma membrane). We have undertaken attempts to investigate CVB3 replication in cells in which pairs of Arfs were knocked down by RNA interference. Unfortunately, in our hands, these combined knockdowns were rather toxic to the cells. Further experiments are required to better understand the possible roles of the different Arf isoforms in enterovirus RNA replication.

In conclusion, this study confirms and extends a crucial role for GBF1 in enterovirus RNA replication. The precise biochemical contribution of GBF1 to virus replication, whether for a membrane-modifying activity that serves to accumulate membrane vesicles or for a downstream step that is important for viral RNA replication, remains to be established.

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