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Effects of Picornavirus 3A Proteins on Protein Transport and GBF1-Dependent COP-I Recruitment$^\nabla$

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The 3A protein of the coxsackievirus B3 (CVB3), an enterovirus that belongs to the family of the picornaviruses, inhibits endoplasmic reticulum-to-Golgi transport. Recently, we elucidated the underlying mechanism by showing that CVB3 3A interferes with ADP-ribosylation factor 1 (Arf1)-dependent COP-I recruitment to membranes by binding and inhibiting the function of GBF1, a guanine nucleotide exchange factor that is required for the activation of Arf1 (E. Wessels et al., Dev. Cell 11:191–201, 2006). Here, we show that the 3A protein of poliovirus, another enterovirus, is also able to interfere with COP-I recruitment through the same mechanism. No interference with protein transport or COP-I recruitment was observed for the 3A proteins of any of the other picornaviruses tested here (human rhinovirus [HRV], encephalomyocarditis virus, foot-and-mouth disease virus, and hepatitis A virus). We show that the 3A proteins of HRV, which are the most closely related to the enteroviruses, are unable to inhibit COP-I recruitment, due to a reduced ability to bind GBF1. When the N-terminal residues of the HRV 3A proteins are replaced by those of CVB3 3A, chimeric proteins are produced that have gained the ability to bind GBF1 and, by consequence, to inhibit protein transport. These results show that the N terminus of the CVB3 3A protein is important for binding of GBF1 and its transport-inhibiting function. Taken together, our data demonstrate that the activity of the enterovirus 3A protein to inhibit GBF1-dependent COP-I recruitment is unique among the picornaviruses.

Picornaviruses are small viruses with a single-stranded RNA genome of positive polarity. The picornavirus family contains the enteroviruses (e.g., poliovirus [PV], human enterovirus A [HEV-A], HEV-B, which include the coxsackie B viruses [CVB], HEV-C, and HEV-D), rhinoviruses (e.g., human rhinovirus [HRV]), cardioviruses (e.g., encephalomyocarditis virus [EMCV]), aphthoviruses (e.g., foot-and-mouth disease virus [FMDV]), hepatoviruses (e.g., hepatitis A virus [HAV]), parechoviruses, erboviruses, kobuviruses, and teschovirus (43). They share essentially one common genome organization (43). The viral genome contains one single open reading frame that encodes a large polyprotein. The polyprotein is processed by virus-encoded proteases to generate the individual structural and nonstructural proteins, as well as some relatively stable cleavage intermediates. Many functions have been connected to the nonstructural proteins, but their exact role in the viral life cycle is still not fully understood (33).

One of the nonstructural proteins is the 3A protein, a small hydrophobic membrane protein. Among the picornaviruses, the 3A protein of the enteroviruses is the best studied. Several mutations in the enterovirus 3A protein were shown to cause defects in viral RNA replication (4, 23, 28, 46, 50, 51). Moreover, amino acid changes in the 3A protein have been shown to alter host range and tropism of enterovirus, HRV, and FMDV, although it is not known how this is mediated (2, 27, 31, 38).

Finally, the enterovirus 3A proteins inhibit endoplasmic reticulum (ER)-to-Golgi transport (16, 50). This inhibition is not absolutely required for replication in tissue culture cells. Instead, the results of several in vitro and in vivo studies suggest that the inhibition of protein transport plays a role in the evasion of immune responses (10, 14, 36, 49).

We recently identified the mechanism by which the CVB3 3A protein inhibits ER-to-Golgi transport (49). Bidirectional transport between the ER and Golgi depends on COP-II and COP-I coat complexes (1, 42). Recruitment of the COP-I coat to membranes is regulated by the GTPase ADP-ribosylation factor 1 (Arf1) (18). Arf1 is converted from its inactive, GDP-bound state to an active, GTP-bound state by guanine nucleotide exchange factors, like GBF1 (17). We showed that CVB3 3A blocks Arf1 activation by interacting with GBF1 and trapping it on membranes (49). As a result, COP-I can no longer be recruited to membranes and protein transport is inhibited.

In addition to this transport-inhibiting function, the PV 3A protein, and also the 3CD protein, was recently shown to recruit Arf proteins to membranes when expressed alone in an in vitro system using HeLa cell extracts (3). This activity has been proposed to be involved in the recruitment of Arf to the viral replication complexes. How the 3A protein can be involved in two such seemingly opposed functions (i.e., inhibition of transport by inhibiting Arf activation on the one hand and replication complex formation by recruiting Arf to membranes on the other hand) is yet unknown, but both observations clearly indicate that the Arf machinery is an important target of the enterovirus 3A protein.

This study focuses on the possible transport-inhibiting func-

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TABLE 1. Primers for generation of 3A-Myc fusion proteins of several picornaviruses[a]

Primer no.	F/R	3A	Sequence
389.1a	F	PV1	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG GGA CCA CTC CAG TAC AAA GAC-3′
389.2	R	PV1, C3PV ch.	5′-GGG GGG *GGA TCC* CTG GTG TCC AGC AAA CAG TTT-3′
389.3	F	HRV14	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG GGA CCA GTG TAT AAA GAT TTA-3′
389.4	R	HRV14, C3H14 ch.	5′-GGG GGG *GGA TCC* TTG AGT TTG AGC AAA CAA TTT-3′
389.5	F	HRV2	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG GGG CCA ATT GAT ATG AAA AAC-3′
389.6	R	HRV2, C3H2 ch.	5′-GGG GGG *GGA TCC* CTG TAA TGT GCA AAA AAG TTT-3′
389.7	F	EMCV	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG GGG CCT GTT GAT GAA GTT AGC-3′
389.8	R	EMCV	5′-GGG GGG *GGA TCC* CTG TTC TTG CTC ATC CAG CTG-3′
389.9	F	FMDV	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG ATC TCA ATT CCT TCT CAA AAA-3′
389.10	R	FMDV	5′-GGG GGG *GGA TCC* TTC AGC TTG TGG TTG CTC CTC-3′
389.11a	F	HAV	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG GGA ATT TCA GAT GAC AAT GCA-3′
389.12	R	HAV	5′-GGG GGG *GGA TCC* TTC AGC TGG GAT TGG TTC CTC-3′
389.16	F	C3H14 ch.	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG GGA CCA CCA GTA TAC AGA GAG ATC AAA ATT AGC GTT GCA CCA GAG ACA CCA CCT CCA GAA TGT ATC AAC-3′
389.14	F	C3H2 ch.	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG GGA CCA CCA GTA TAC AGA GAG ATC AAA ATT AGC GTT GCA CCA GAG ACA CCA CCA CCA CCT GCT ATT ACT-3′
389.15	F	C3PV ch.	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG GGA CCA CCA GTA TAC AGA GAG ATC AAA ATT AGC GTT GCA CCA GAG ACA CCC CCT CCT GAA TGT ATC AAT-3′
389.17	F	HRV14 3A-E8K	5′-GGG GGG <u>GTC GAC</u> GCC ACC ATG GGA CCA GTG TAT AAA GAT **CTT AAG** ATT GAT GTT TGC AAC ACA CCA-3′

[a] F, forward primer, with SalI site underlined; R, reverse primer, with BamHI site shown in italics. The BfrI site is shown in boldface for rapid identification of the E8K mutation. C3PV ch., CVB3/PV1 3A chimeric protein; C3H14 ch., CVB3/HRV14 3A chimeric protein; C3H2, CVB3/HRV2 3A chimeric protein.

tion of the 3A proteins of other picornaviruses. This function of the enterovirus 3A protein is not necessarily conserved among the different genera, since the nomenclature of the picornavirus proteins is solely based on their position in the viral RNA genome. Here, we investigated the putative transport-inhibiting function of the picornavirus 3A proteins, with a special focus on their ability to inhibit GBF1-dependent COP-I recruitment to membranes.

MATERIALS AND METHODS

Cells and DNA transfections. Buffalo green monkey (BGM) kidney cells were grown in minimal essential medium (Gibco) supplemented with 10% fetal bovine serum. Cells were grown at 37°C in a 5% CO_2 incubator. BGM cell monolayers were grown to subconfluency on coverslips in 24-well plates for immunofluorescence assays or in glass-bottom dishes (WillCo Wells BV) for fluorescence recovery after photobleaching (FRAP) experiments and then transfected with 0.5 μg or 1 μg of plasmid DNA, respectively. Transfections were performed using FuGENE 6 (Roche) according to the manufacturer's instructions. Cells were grown at 37°C until further analysis, unless stated otherwise.

Plasmids. (i) p3A-Myc constructs. The plasmids coding for the different picornavirus 3A proteins were generated by PCR amplification using specific primers for the 3A coding sequences (Table 1). The forward and reverse primers introduced SalI and BamHI sites, respectively. The PCR products were cloned in p2B-Myc (11), from which the 2B coding sequence was removed using the restriction enzymes SalI and BamHI. The following templates were used for

PCR: pXPA (for PV) (40), pWR3.26 (for HRV14) (32), pHRV2/1 (for HRV2) (19), pM16.1 (for EMCV, strain Mengo virus) (20), pMR15 (for FMDV) (41), and pHAV/7 (for HAV) (9). Sequence analysis showed that all PCR products contained correct sequences.

(ii) pCFP-3A constructs. pCFP-fusion constructs were generated by cloning the 3A coding sequences of the different picornavirus 3A-Myc constructs (using SalI/BamHI) into a modified EGFP-C3 vector cut with XhoI/BamHI. The green fluorescent protein (GFP) coding sequence was subsequently replaced with the cyan fluorescent protein (CFP) coding sequence using the restriction enzymes NheI and SspBI.

(iii) pGBT9-3A constructs. pGBT9-3A (CVB3) was described before (49). The other constructs were generated by PCR amplifying the 3A coding sequences of the different picornaviruses and cloning these PCR products in pGBT9 using the restriction enzymes EcoRI and BamHI. The primers are depicted in Table 2.

(iv) pVSVG-GFP. The plasmid pVSVG-GFP (47), which carries the *ts*045 vesicular stomatitis virus glycoprotein (VSVG) fused to enhanced green fluorescent protein (EGFP) at its C terminus, was kindly provided by P. Keller and K. Simons (Max Planck Institute of Molecular Biology and Genetics, Dresden, Germany).

(v) pYFP-GBF1. The pYFP-GBF1 plasmid was described previously (37).

Immunofluorescence and VSVG trafficking. The effects of different picornavirus 3A proteins on COP-I membrane association were studied by staining 3A-Myc-expressing cells with a monoclonal anti-Myc antiserum (Sigma; 1:200 diluted) and a polyclonal anti-COP-I (against α- and γ-COP) antiserum (from K. Frey and F. Wieland, Biochemie-Zentrum Heidelberg, Germany; 1:200 diluted). The subcellular localization of VSVG-GFP was determined as described previously (11). Briefly, BGM cells expressing either VSVG-GFP alone or VSVG-

TABLE 2. Primers for generation of pGBT9-3A constructs of several picornaviruses[a]

Primer no.	F/R	3A	Sequence
389.18	F	PV1	5′-GGG GGG <u>GAA TTC</u> AGT ATG GGA CCA CTC CAG TAC AAA-3′
389.19	R	PV1	5′-GGG GGG *GGA TCC* CTA GAT GTT CCT TTC TGT TTG-3′
389.20	F	HRV14	5′-GGG GGG <u>GAA TTC</u> AGT ATG GGA CCA GTG TAT AAA GAT-3′
389.21	R	HRV14	5′-GGG GGG *GGA TCC* CTA CAT AGC CCT TTC TAT GTT-3′
389.22	F	HRV2	5′-GGG GGG <u>GAA TTC</u> AGT ATG GGG CCA ATT GAT ATG AAA-3′
389.23	R	HRV2	5′-GGG GGG *GGA TCC* CTA TTC TGC TGG AAT TAT CCA-3′

[a] F, forward primer, with the EcoRI site underlined; R, reverse primer, with the BamHI site shown in italics.

GFP together with the picornavirus 3A-Myc proteins were incubated at 40°C. After a 2-h temperature shift to 32°C, the cells were fixed and stained with anti-Myc antiserum to detect the 3A-Myc proteins and a polyclonal anti-EGFP antiserum (raised against recombinant GST-EGFP; 1:200 diluted) to enhance the VSVG signal. The samples were analyzed using a Leica DMR microscope, and pictures were taken under a Leica TCS NT microscope (Leica Lasertechnik GmbH, Heidelberg, Germany). Fluorescent conjugates were obtained from Molecular Probes.

FRAP experiments. The FRAP experiments were performed on a Zeiss LSM510Meta confocal microscope (Carl Zeiss GmbH, Jena, Germany) as described previously (49). Briefly, FRAP measurements were performed on time-lapse series that were taken at 37°C at a rate of 1 frame per s using a 63×, 1.4 numerical aperture objective and pinhole settings such that <2-μm optical slices were imaged. Cells were selected on the basis of coexpression of CFP-3A and yellow fluorescent protein (YFP)-GBF1. Regions of interest were selectively photobleached using the 488-nm and 514-nm line at 100% transmission.

Yeast two-hybrid analysis. Yeast two-hybrid analysis was performed in *Saccharomyces cerevisiae* strain AH109 (Clontech) as described previously (49). Briefly, the pGADT7 and pGBT9 fusion constructs were transformed into yeast by the lithium acetate method using carrier DNA and plated on nonselective plates (lacking leucine and tryptophan). After growth, the colonies were transferred to selective plates (lacking histidine or adenine).

Statistical analysis. Data are presented as mean values ± standard errors of the means. Differences were tested for significance by the Student t test.

RESULTS AND DISCUSSION

Only enterovirus 3A proteins, but none of the other picornavirus 3A proteins, interfere with COP-I recruitment to membranes. We investigated the ability of the 3A proteins of CVB3, PV1, HRV2 (a group A HRV), HRV14 (a group B HRV), EMCV, FMDV, and HAV to interfere with COP-I recruitment to membranes (Fig. 1A). Since antibodies against all of the picornavirus 3A proteins used in this study were not readily available, we generated C-terminal Myc fusions of the 3A proteins. BGM cells expressing the 3A proteins were stained with antibodies against the Myc tag and COP-I. Due to differences in transfection efficiencies of the different constructs, we assayed the effects of the picornavirus 3A proteins on COP-I localization by examining similar, large numbers of individual cells with comparable expression levels. 3A-expressing cells were divided into three categories based on their COP-I localization: (i) COP-I-positive intact Golgi structures, (ii) COP-I-positive dispersed membrane structures, and (iii) COP-I exclusively in the cytosol. In nontransfected control cells, COP-I was typically localized to Golgi membranes (Fig. 1A). In the majority of cells expressing CVB3 3A, COP-I was redistributed to the cytosol and the 3A protein localized in the ER and a post-ER compartment that was partially positive for COP-II, as described previously (Fig. 1A and B) (49, 50). COP-I localization in cells expressing PV1 3A varied among cells. In the majority of the PV1 3A-expressing cells, COP-I recruitment was strongly affected. In about half of these cells, COP-I was redistributed to the cytosol. In these cells, PV1 3A showed a localization pattern that was similar to that of CVB3 3A. In the other half of the cells, which were generally cells with lower PV1 3A expression levels, 3A and COP-I were associated with dispersed membrane structures (Fig. 1A and B). Remarkably, this was not observed in cells expressing lower levels of CVB3 3A, suggesting that this protein is a little more potent than PV 3A in inhibiting COP-I recruitment to membranes.

No effect on COP-I recruitment and localization was observed upon expression of the 3A proteins of HRV14 or

HRV2, both of which localized to the Golgi complex (Fig. 1A and B). In addition, no effect on COP-I recruitment was observed in cells expressing the 3A proteins of EMCV and FMDV, both of which localized in the ER (as shown by colocalization with an ER marker [data not shown]), or HAV, which localized to an unidentified compartment that showed no overlap with markers for the ER, Golgi, ER exit sites, endosomes, lysosomes, and mitochondria (Fig. 1A and B).

We also tested the effects of these picornavirus 3A proteins on intracellular transport. To this end, we monitored the trafficking of the temperature-sensitive mutant of VSVG, a well-known marker for protein trafficking that at 40°C accumulates in the ER but after shifting to the permissive temperature (32°C) is transported to the plasma membrane. In the majority of cells expressing CVB3 3A or PV1 3A, transport of VSVG out of the ER was blocked at the permissive temperature (Fig. 1C). This strongly suggests that transport was also inhibited in the PV1 3A-expressing cells in which COP-I was observed at dispersed membranes. None of the other picornavirus 3A proteins tested interfered with VSVG trafficking to the plasma membrane (Fig. 1C). Together, these data indicate that only the 3A proteins of CVB3 and PV1, both belonging to the genus *Enterovirus*, inhibit transport and do so by interfering with COP-I recruitment. While this work was in progress, the group of Kirkegaard reported similar results (8). Those authors showed that only the 3A proteins of PV1, PV3, and CVB3, but not those of HRV14, enterovirus type 71, HAV, Theiler's virus (a cardiovirus), and FMDV, were able to inhibit ER-to-Golgi transport, as monitored by the acquisition of Golgi-specific modifications resulting in endoglycosidase H resistance of the VSVG protein. Our finding that the 3A proteins of CVB3 and PV1 interfere with COP-I recruitment to membranes provides a plausible explanation for their observations. Moreover, our finding that the 3A proteins of the other picornaviruses do not interfere with trafficking of VSVG to the plasma membrane demonstrates that these proteins have no inhibitory effect on transport through or beyond the Golgi complex. The inability to block transport of FMDV 3A, EMCV 3A, and HAV 3A is likely related to the large differences in amino acid sequence between those proteins and the enterovirus 3A protein (data not shown).

The HRV 3A proteins do not trap GBF1 on membranes. Enteroviruses and HRVs are very closely related in sequence and functional terms, and it has even been suggested that they be combined into one genus (G. Stanway et al., Europic 2005, Lunteren, The Netherlands, abstr. A05). Alignment of the 3A proteins of CVB3, PV1, HRV14, and HRV2 (Fig. 2A) showed that the 3A protein of PV1 shares 51% identity and 85% homology with CVB3 3A. HRV14 3A shares 50% identity and 81% homology with CVB3 3A and 49% identity and 82% homology with PV1 3A. Notwithstanding this high sequence homology, the transport-inhibiting activity is not conserved in the HRV 3A proteins.

We reasoned that the inability of the HRV 3A proteins to block COP-I recruitment to membranes is most likely due to their inability to inhibit the function of Arf1, the GTPase that is responsible for COP-I recruitment. We have shown that CVB3 3A interferes with Arf1 activation by inhibiting the dynamic behavior and function of GBF1 (49), a guanine nucleotide exchange factor for Arf1 that under normal conditions
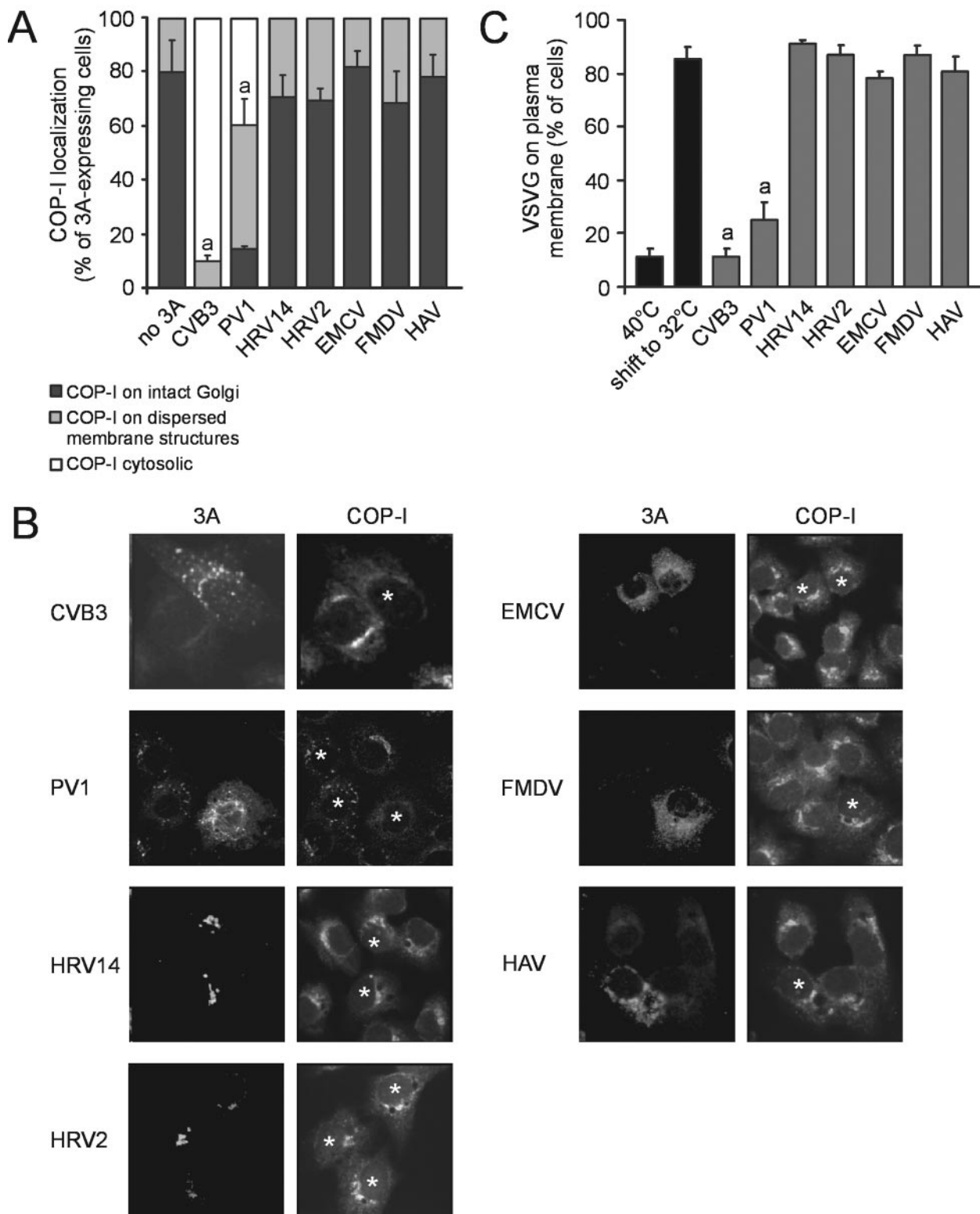
FIG. 1. Effects of picornavirus 3A proteins on COP-I recruitment to membranes and VSVG trafficking. (A) COP-I distribution in 3A-expressing cells. The bars show the means ± standard errors of the means (SEM) of the percentage of cells in which COP-I was redistributed to the cytosol, or localized at intact Golgi or dispersed membrane structures, as calculated from at least 400 cells, which were counted in groups of ~100 cells in two independent experiments. As a control, we determined COP-I localization in cells expressing the CVB3 2B protein (no 3A). (B) BGM cells expressing Myc-tagged CVB3, PV1, HRV14, HRV2, EMCV, FMDV, or HAV 3A proteins were stained for the Myc tag and COP-I (α/γ-COP). Asterisks indicate 3A-positive cells. (C) The percentage of cells in which the VSVG protein was localized to the plasma membrane. At 40°C, VSVG is improperly folded and as a consequence retained in the ER. Upon shifting to the permissive temperature (32°C), VSVG is correctly folded and transported to the plasma membrane. The graph shows the means ± SEM of the percentage of cells in which VSVG localized at the plasma membrane, calculated from at least 200 cells, which were counted in groups of ~50 cells in two independent experiments. "a" indicates result significantly different from control cells without 3A (calculated by using the Student t test; $P < 0.05$).
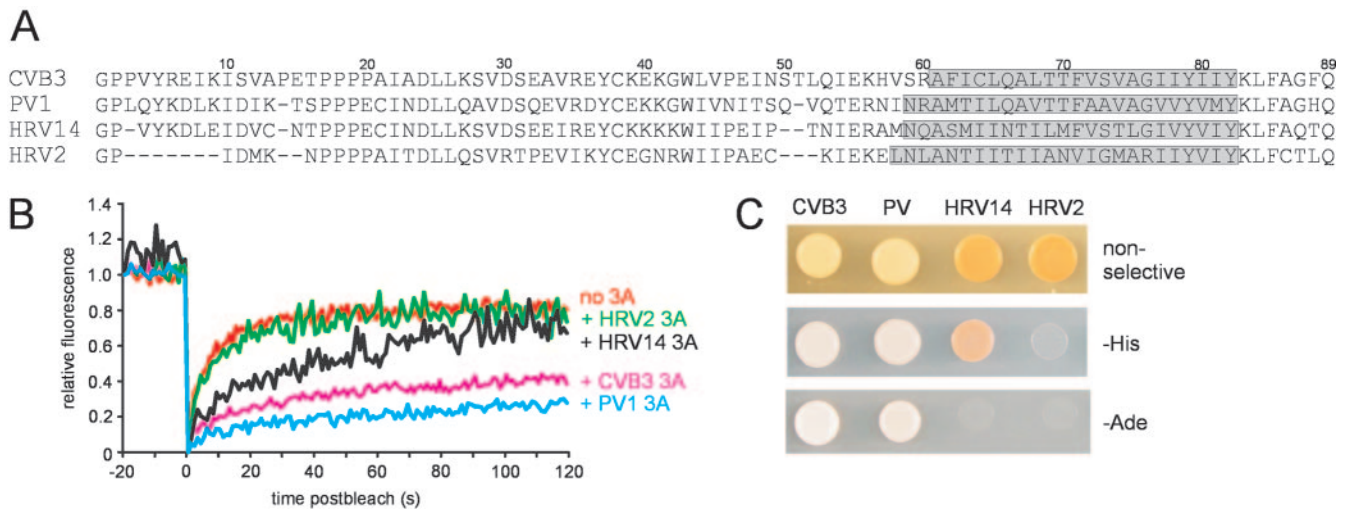
FIG. 2. The HRV 3A proteins have a reduced ability to bind GBF1 and inhibit its function. (A) Alignment of enterovirus and HRV 3A proteins. Gray boxes indicate the hydrophobic regions of the 3A proteins, as predicted using the Kyte and Doolittle method. (B) FRAP traces of cells expressing YFP-GBF1 either alone (no 3A) or together with CFP fusion proteins of the 3A proteins of CVB3, PV, HRV14, or HRV2. The traces in the graphs show the average recovery ($n > 10$ cells) from at least two independent experiments. The fluorescence intensity before bleaching was normalized to 1, and the fluorescence intensity directly after bleaching was normalized to 0. The fluorescence intensity was corrected for bleaching of the cell during imaging and for background fluorescence. (C) Interaction of the 3A proteins of CVB3, PV1, HRV14, and HRV2 with the N-terminal part of GBF1 as assayed by yeast two-hybrid analysis. Upper, middle, and lower panels show growth of yeast on nonselective medium (leucine- and tryptophane-deficient medium), selective medium lacking histidine (-His), and selective medium lacking adenine (-Ade), respectively.

rapidly cycles on and off membranes (37). We studied the dynamics of GBF1 in cells coexpressing YFP-GBF1 and CFP fusions of the 3A proteins of CVB3, PV1, HRV14, and HRV2 by FRAP analysis (Fig. 2B). In control cells expressing YFP-GBF1, which localized mainly at the ER but to some extent also at the Golgi, the fluorescence recovered rapidly to the bleached region. In cells coexpressing CVB3 or PV1 3A and GBF1, which colocalized on dispersed membranes (49), the YFP-GBF1 fluorescence recovered very slowly and only to a limited extent, indicating that these 3A proteins trap GBF1 on the dispersed membranes (Fig. 2B). In cells coexpressing HRV2 3A and GBF1, which colocalized mainly in the ER and at dispersed membranes, fluorescence recovered rapidly to the bleached region with a kinetics similar to that observed in control cells. Recovery of fluorescence was also observed in cells coexpressing HRV14 3A and GBF1, which colocalized mainly at Golgi structures and dispersed membranes, but the kinetics was slower than that observed in control cells and HRV2 3A-expressing cells (Fig. 2B).

It is likely that the reduced ability of the HRV 3A proteins to trap GBF1 on membranes is due to a reduced ability of these proteins to interact with GBF1. To study this, we tested the binding between the 3A proteins and GBF1. Previously, we had shown an interaction in a yeast two-hybrid system between the cytosolic part of CVB3 3A (3A[1-60]) and the N-terminal part of GBF1 (i.e., the region upstream of the catalytic Sec7 domain) (49). We used this yeast two-hybrid system to test whether the corresponding regions of the 3A proteins of PV1, HRV2, and HRV14 were able to bind to GBF1 (Fig. 2C). A strong interaction between the two proteins results in the growth of white colonies on selective media lacking histidine or adenine, whereas a weak interaction results in the growth of red colonies on selective medium lacking histidine. Both the

CVB3 and PV1 3A proteins strongly interacted with GBF1 (Fig. 2C). In contrast, HRV14 3A showed a weak interaction with GBF1, whereas no interaction between HRV2 3A and GBF1 was detected (Fig. 2C). Together, it can be concluded that the inability of the HRV 3A proteins to interfere with COP-I recruitment to membranes is a result of their reduced ability to bind GBF1.

Importance of the N terminus of 3A to inhibit protein transport. All human enteroviruses sequenced to date contain a residue with a positive charge at position 9 (numbering refers to CVB3 3A). Strikingly, the HRV14 3A protein contains a negatively charged residue at the corresponding position. Choe et al. showed that substitution of this negatively charged residue for a positively charged residue conferred a partial ability to the HRV14 3A protein to inhibit protein secretion (8). To investigate whether this single difference in the N terminus of HRV14 3A could increase its ability to inhibit GBF1, we substituted the negatively charged Glu in HRV14 3A for a positively charged Lys (HRV14 3A-E8K). The HRV14 3A-E8K mutant showed an increased ability to interfere with VSVG transport and COP-I recruitment to membranes compared to HRV14 3A wild type (wt) (Fig. 3A and B). In ∼35% of the cells expressing HRV14 3A-E8K, COP-I was redistributed to the cytoplasm. In these cells, HRV 3A-E8K showed a localization pattern that was similar to that of CVB3 3A (Fig. 3C). HRV14 3A-E8K was expressed to similar levels as HRV14 3A wt, making it unlikely that its increased ability to block transport is due to a higher expression level.

Next, we investigated whether the E8K mutation also increased the ability of HRV14 3A to bind GBF1. Indeed, the HRV14 3A-E8K mutant showed a strong interaction with GBF1 in yeast two-hybrid analysis, whereas HRV14 3A wt showed only a weak interaction with GBF1 (Fig. 3D). In cells
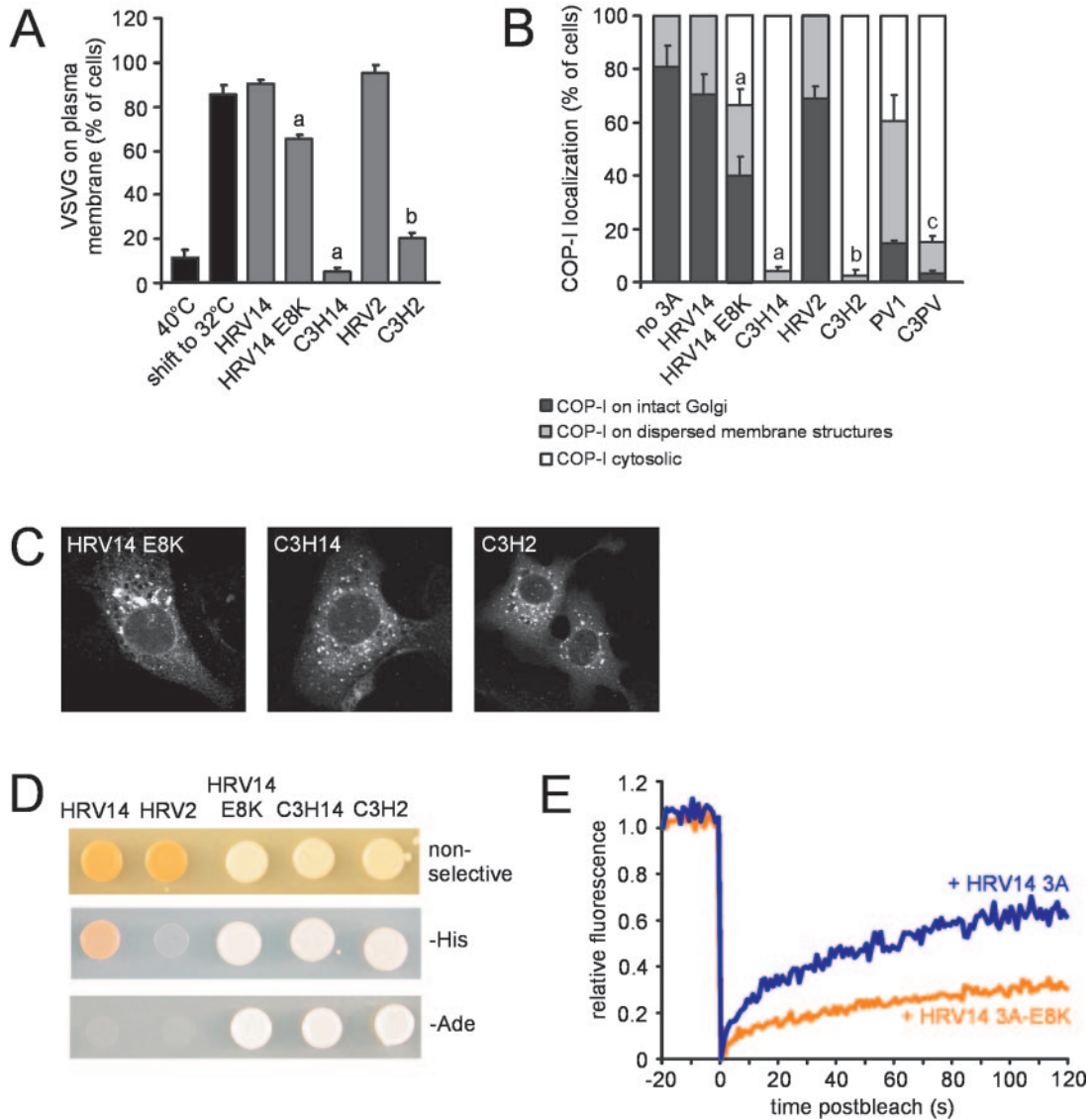
FIG. 3. The N terminus of 3A is very important for its transport-inhibiting function. (A) The percentage of cells in which the VSVG protein was localized at the plasma membrane. The percentages were calculated as described in the legend to Fig. 1. (B) COP-I localization in cells expressing HRV14 3A-E8K, C3H14 3A, C3H2 3A, or C3PV 3A. The percentages were calculated as described in the legend to Fig. 1. (C) BGM cells expressing Myc-tagged mutant or chimeric 3A proteins (HRV14 3A-E8K, C3H14, and C3H2) were stained for the Myc tag to show their localization. (D) Interaction of the mutant or chimeric 3A proteins (HRV14 3A-E8K, C3H14, and C3H2) with the N-terminal part of GBF1 as assayed by yeast two-hybrid analysis. The results are depicted as described in the legend to Fig. 2. (E) FRAP traces of cells coexpressing YFP-GBF1 and CFP fusion proteins of HRV14 3A (wild-type or E8K). Traces were calculated as described in the legend for Fig. 2. C3H14, CVB3/HRV14 3A chimeric protein; C3H2, CVB3/HRV2 3A chimeric protein; C3PV, CVB3/PV1 3A chimeric protein. a, significantly different from HRV14 3A wt-expressing cells; b, significantly different from HRV2 3A wt-expressing cells; c, significantly different from PV1 3A wt-expressing cells (calculated with Student's t test; $P < 0.05$).

coexpressing HRV14 3A-E8K and GBF1, which colocalized on dispersed membranes, YFP-GBF1 fluorescence recovered very slowly and only to a limited extent, indicating that the HRV14 3A-E8K mutant is much more potent for trapping GBF1 on membranes than the HRV14 3A wt protein (Fig. 3E). Together, these results indicate that a single substitution in the N terminus of the HRV14 3A protein improved its ability to bind and inhibit GBF1 and thereby conferred the ability to inhibit protein transport.

It may seem remarkable that the mutation E8K conferred only a partial ability to interfere with protein trafficking and COP-I recruitment to HRV14 3A, whereas the HRV14 3A-E8K mutant interacted with GBF1 and trapped it on membranes as efficiently as CVB3 3A (which is far more potent in inhibiting COP-I recruitment to membranes and VSVG trafficking). These differences may be explained by the semiquantitative nature of the yeast-two hybrid test and the selective analysis of cells in the FRAP experiments. In the FRAP experiments only cells expressing higher levels of CFP-3A were taken into account, since cells with low or moderate 3A ex-

pression were in general poorly visible due to the relatively low fluorescence yield of CFP.

The above-mentioned HRV14 3A-E8K mutation provides only a partial ability to interfere with COP-I recruitment and to inhibit VSVG transport. We asked ourselves whether substitution of other residues in the N terminus might further increase the transport inhibiting activity of the HRV14 3A protein. To investigate this, we constructed a chimeric protein in which the N-terminal 14 amino acids (aa) of HRV14 3A were replaced by the corresponding 16 aa of CVB3 3A (Fig. 2A, note that the N terminus of HRV14 3A lacks two residues compared to that of CVB3 3A). The chimeric CVB3/HRV14 3A protein (C3H14), which was expressed at similar levels as HRV14 3A wt, was very potent in interfering with VSVG transport and COP-I recruitment to membranes (Fig. 3A and B) and showed a localization pattern that was similar to that of CVB3 3A (Fig. 3C). As expected, the C3H14 3A chimeric protein showed a strong interaction with GBF1 (Fig. 3D). Thus, substitution of the N terminus of HRV14 3A for the corresponding region of CVB3 3A further improved the ability to interfere with transport and COP-I recruitment compared to the HRV14 3A-E8K mutant. Taken together, the results with the mutant and chimeric HRV14 3A proteins show that the N terminus of 3A is very important for the protein transport inhibiting function.

The HRV2 3A protein shares 38%, 37%, and 40% identity with CVB3, PV1, and HRV14 3A, respectively (Fig. 2A). Among these four viruses, the N terminus of HRV2 3A in particular differs strongly from the N termini of the three other 3A proteins: it lacks 9 aa in its N terminus compared to CVB3 3A. Also, in the rest of the HRV2 3A protein, there are some major differences compared to the other 3A proteins (Fig. 2A). To investigate whether these latter differences affect a putative transport-inhibiting function, we constructed a chimeric protein in which the N-terminal 7 aa of HRV2 3A were replaced by the corresponding 16 aa of CVB3 3A. The ability of this C3H2 3A chimeric protein to interfere with VSVG transport and COP-I recruitment to membranes was significantly increased compared to the wt HRV2 3A and was similar to that of CVB3 3A and the above-described C3H14 chimeric 3A (Fig. 3A and B). Also, the localization of C3H2 3A was similar to that of CVB3 3A and C3H14 3A, and C3H2 3A showed a strong interaction with GBF1 (Fig. 3C and D). Thus, although the HRV2 3A protein shows a number of distinct differences compared to the 3A proteins of enteroviruses and HRV14, replacement of its N terminus with that of CVB3 3A provides the capability to inhibit protein transport with high efficiency.

We also constructed a chimeric PV1 3A protein containing the N-terminal 16 aa of CVB3 (C3PV 3A). This chimeric protein was generated based on the observation that COP-I was redistributed to the cytosol in almost all CVB3 3A-expressing cells, whereas in about half of the PV1 3A-expressing cells only an intermediate effect on COP-I was observed (i.e., some COP-I was found on dispersed membrane structures) (Fig. 1A). This finding suggested that CVB3 3A is (a little bit) more potent in inhibiting COP-I recruitment to membranes than PV1 3A. Indeed, we found that a chimeric PV1 3A protein containing the N-terminal 16 aa of CVB3 was much more potent in redistributing COP-I to the cytoplasm than PV1 3A (Fig. 3B), supporting the idea that small differences in the N

terminus make the CVB3 3A more active in inhibiting transport. Our findings that PV1 3A was equally potent as CVB3 3A in interacting with GBF1 in the yeast two-hybrid analysis and that GBF1 is trapped on membranes equally efficiently by CVB3 3A and PV1 3A do not argue against this idea, because of the same reasons as described above for the HRV-E8K mutant (i.e., semiquantitative nature of the yeast two-hybrid analysis and selective analysis of high-expressing cells in FRAP experiments).

Implications for the viral life cycle. As shown above, the N terminus of 3A is important for its protein transport-inhibiting function. The 3A proteins of CVB3, PV1, and PV3 inhibit protein transport (8, 16, 50). CVB3 belongs to the HEV-B species (which includes all CVB and echoviruses, CVA9, and some unassigned enterovirus serotypes). The N termini of the 3A proteins of all members of this species are nearly identical, suggesting that all of them inhibit protein transport. The high similarity between the 3A proteins of the HEV-B and HEV-D species (which include enterovirus types 68 and 70) suggests that the 3A proteins of these latter viruses also inhibit transport. The strong resemblance between the N termini of the 3A proteins of PV and enteroviruses belonging to the HEV-C species (which include a number of CVA) suggests that the transport-inhibiting function is also conserved in HEV-C 3A proteins. In contrast, the 3A protein of enterovirus type 71, a member of the HEV-A species (which furthermore includes enterovirus types 76, 89, 90, and 91 and a number of CVA), was found to be unable to inhibit transport (8). Remarkably, this virus (and also all other HEV-A members and the bovine and porcine enteroviruses sequenced to date) contain a proline at position 7 in 3A, whereas all other enteroviruses contain a negatively charged residue at this position. It remains to be established whether this amino acid is responsible for the inability of the enterovirus type 71 3A protein to inhibit transport. If so, then it is likely that the other HEV-A members and the nonhuman enteroviruses mentioned above are also impaired in this function.

The inhibition of protein transport by PV and CVB3 has been suggested to be important for the suppression of immune responses, such as cytokine secretion and major histocompatibility complex (MHC) exposure (10, 14, 36, 49). Picornavirus 3A proteins that are unable to inhibit protein transport likely cannot aid in the inhibition of trafficking of cytokines and MHC molecules. Possibly, transport inhibition by other viral proteins aids in this immune evasion. The 2B proteins of enterovirus (12, 16) and HRV14 (A. S. de Jong and F. J. M. van Kuppeveld, unpublished data) as well as the 2BC protein of FMDV, have also been reported to inhibit protein transport (35). Whether EMCV and HAV also express proteins that can inhibit protein transport is unknown. Furthermore, it should be emphasized that the inhibition of protein transport is not the only viral activity that determines immune evasion. Several other mechanisms have been described by which picornaviruses can evade immune responses. Several picornaviruses shut off host cell transcription (enterovirus, HRV, and FMDV) and translation (enterovirus, HRV, EMCV, and FMDV), thereby interfering with the production of host cell proteins with antiviral functions (7, 13, 21, 24, 45, 48). Moreover, enteroviruses, HRV, and EMCV inhibit nuclear import pathways, possibly to prevent signal transduction into the nucleus

(25, 26, 34). The consequence of the inability of picornavirus 3A proteins to inhibit protein transport for the evasion of immune responses is hence difficult to define.

All picornaviruses replicate their RNA genome at modified membranes that accumulate in the cytosol of infected cells. However, the mechanisms by which the replication complexes are formed differ among the different picornaviruses, as shown by different sensitivities towards the inhibitory action of the drug brefeldin A (BFA). Enterovirus, rhinovirus, and HAV replication is blocked by BFA, whereas BFA has little effect on replication of EMCV and FMDV (6, 22, 29, 39). For enteroviruses, it has been suggested that these rearranged membranes are derived from the secretory pathway through the action of the viral 2BC protein (5), possibly in conjunction with the 3A protein (44). Alternatively, or in addition, it has been suggested that enteroviruses subvert constituents of the cellular autophagy pathway to form membrane scaffolds for viral RNA replication through the combined actions of the 2BC and 3A proteins (30). Subversion of constituents of the autophagy pathway was also observed in cells infected with HRV (30). The observation that enteroviruses carrying a mutation in 3A that rendered it defective in inhibiting transport replicated with wild-type characteristics suggested that, irrespective of the underlying mechanism, the 3A-mediated transport inhibition is not required for the accumulation of the modified membranes (15, 50). HRV infection leads to similar membrane rearrangements as observed in enterovirus-infected cells (27). The finding that the 3A protein of HRV is unable to inhibit transport further supports the idea that the accumulation of the membranes at which viral replication takes place does not rely on transport inhibition by 3A.

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