Reticulon 3 Binds the 2C Protein of Enterovirus 71 and Is Required for Viral Replication

Received for publication, December 5, 2006 Published, JBC Papers in Press, December 20, 2006, DOI 10.1074/jbc.M611145200

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Enterovirus 71 is an enterovirus of the family Picornaviridae. The 2C protein of poliovirus, a relative of enterovirus 71, is essential for viral replication. The poliovirus 2C protein is associated with host membrane vesicles, which form viral replication complexes where viral RNA synthesis takes place. We have now identified a host-encoded 2C binding protein called reticulon 3, which we found to be associated with the replication complex through direct interaction with the enterovirus 71-encoded 2C protein. We observed that the N terminus of the 2C protein, which has both RNA- and membrane-binding activity, interacted with reticulon 3. This region of interaction was mapped to its reticulon homology domain, whereas that of 2C was encoded by the 25th amino acid, isoleucine. Reticulon 3 could also interact with the 2C proteins encoded by other enteroviruses, such as poliovirus and coxsackievirus A16, implying that it is a common factor for such viral replication. Reduced production of reticulon 3 by RNA interference markedly reduced the synthesis of enterovirus 71-encoded viral proteins and replicative double-stranded RNA, reducing plaque formation and apoptosis. Furthermore, reintroduction of nondegradable reticulon 3 into these knockdown cells rescued enterovirus 71 infectivity, and viral protein and double-stranded RNA synthesis. Thus, reticulon 3 is an important component of enterovirus 71 replication, through its potential role in modulation of the sequential interactions between enterovirus 71 viral RNA and the replication complex.

Enterovirus 71 (EV71)² is a member of the Picornaviridae family and contains a single-stranded RNA genome of positive polarity. The genome of human enteroviruses is about 7.5 kilobases. It encodes a single polyprotein that is proteolytically cleaved to give rise to various structural and nonstructural viral proteins. The human enterovirus family is classified into five species: the poliovirus (PV), coxsackievirus A, coxsackievirus B, echovirus, and other enteroviruses (1). Their genomes are highly homologous and thus share very similar biochemical properties. One of their universal features is that infection with positive-stranded RNA viruses results in a range of membrane morphologies, many of which involve complex membrane rearrangements. Vesicular structures generated in picornavirus-infected cells appear to serve as compartments where synthesis of viral RNA takes place. These structures are derived from membranes of different cellular organelles involved in secretory pathways, primarily endoplasmic reticulum (ER) but also the Golgi complex and possibly others (2–5). These membrane rearrangements are clearly triggered by the viral preprotein 2BC and its mature polypeptide 2C (6–8). The vesicles are associated with the above viral proteins (2, 9) and with other viral nonstructural polypeptides (42) and exhibit a rosette-like morphology with elongated replication complexes surrounded by round vesicles (9). They are equipped with all the necessary components of the RNA- and translation-synthesizing machinery. It is not clear how the viral RNA is located to these complexes, and the mechanism by which these vesicles are generated is unknown. Furthermore, it has not been determined whether host-encoded proteins play any role in the formation of these replication complex-associated vesicles.

The 2C protein is one of the most highly conserved of the viral proteins among all picornaviruses. The PV 2C protein is 37.5 kDa and is composed of 329 amino acids containing a central motif characteristic of nucleoside triphosphate binding proteins (10, 11). Sequences present at both the N- and the C-terminal portions of the protein possess nonspecific RNA binding activity in vitro (12, 13). Both 2C and its precursor 2BC appear to be associated with viral RNA in infected cells (9), where the 2C (and 2BC) protein are closely associated with replication complex-associated vesicles (2, 9, 14, 15). They may be involved in the formation or maintenance of these complexes (9). Additionally, the 2C protein, when translated in vitro in rabbit reticulocyte lysates, binds tightly to exogenous microRNA molecules (16).
sominal membranes (16). In these in vitro binding studies, the N-terminal region encompassing amino acids 21–54 was required for membrane binding. This region is associated with a conserved, predicted amphipathic helix between residues 18 and 35 (17). Thus, the N-terminal 2C possesses the ability to bind RNA and cellular membranes. Disruption of this amphipathic region results in defective viral RNA synthesis. However, the precise role of 2C in RNA replication is not defined.

We performed a two-hybrid experiment in yeast to search for cellular proteins that interact with the N-terminal region of the 2C protein of EV71, which might modulate virus activity. We report here the identification of reticulon 3 (RTN3), a member of the reticulon family of proteins, as a binding partner of the viral 2C protein of EV71. There are four members of the reticulon family, all of which contain a highly conserved reticulon homology domain (RHD) of about 188 amino acids, with two putative transmembrane regions separated by a 66-residue loop (18, 19). Moreover, the RTN family is involved in membrane trafficking and maintaining ER shaping (20–23). Because of these interactions and expression patterns, their functions have been postulated to include structural stabilization of the ER network, proapoptotic mechanisms, protein secretion, and transport of constituents of the ER to other compartments (18). However, their functions in relation to viral replication, particularly via interaction with the encoded viral proteins of enterovirus 71, remain unknown. Our data indicate that changes in RTN3 expression in cells modulate EV71 viral double-stranded RNA (dsRNA) synthesis and protein expression markedly. In addition, the 2C proteins of PV and coxsackievirus A16 (CA16) were demonstrated to interact with RTN3. Taken together, our findings provide evidence that the host-encoded RTN3 protein functions as a cellular modifier of enterovirus infection and replication.

**EXPERIMENTAL PROCEDURES**

*Cell Culture, Viruses, and Plaque Assay*—HeLa, HEK-293T, RD, and Vero cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a 5% CO2 incubator. Puromycin at 0.5 μg/ml was used to select and maintain silencing RNA (siRNA) in HeLa cells. The RTN3-retransformed HeLa cells (2A3) were maintained in puromycin at 0.5 μg/ml and G418 at 1 mg/ml. The EV71 and CA16 were from the Clinical Virology Laboratory, Department of Pathology, Chang Gung Memorial Hospital, and PV vRNA (Type 1, Mahoney) was from Dr. Chun-Nan Lee at the School of Medical Sciences, Chang Gung Memorial Hospital, and PV vRNA (Type 1, Mahoney) was from Dr. Chun-Nan Lee at the School of Medical Sciences, Chang Gung Memorial Hospital.

**Plasmids**—The plasmids pGBK7-EV71 2C1 and pGBK7-EV71 2C2 were constructed as baits for yeast two-hybrid screening, in which the enterovirus 71 2C protein N-terminal amino acids 5–43 and 5–124, respectively, were fused to the DNA binding domain of GAL4 in the vector pGBK7. Corresponding cDNA fragments were produced from EV71 cDNA in pCR-XL-TOPO (a gift from Dr. Mei-Shang Ho, Academia Sinica, Taiwan) by PCR amplification using specific primers. The amplified DNA fragments were inserted into pCR2.1-TOPO (Invitrogen) and subsequently subcloned in-frame into NcoI and EcoRI sites of pGBK7 to produce the plasmids pGBK7-EV71 2C1 and pGBK7-EV71 2C2 for two-hybrid screening. The full-length clone of human RTN3 (RTN3A1 with 236 amino acids, accession number AF059524) (25) and its truncated mutants were obtained from a human fetal brain cDNA library by PCR amplification using specific primers and were inserted into pcDNA3.1-Myc-His. The N-terminal 2C proteins (2C1 and 2C2) of enterovirus 71 were cloned into an EcoRI site of pEGFP-C1 to produce the plasmids pEGFP-EV71 2C1 and pEGFP-EV71 2C2. Full-length EV71 2C was amplified from EV71 cDNA in pCR-XL-TOPO and cloned into pEGFP. PV 2C and CA16 2C full-length cDNA fragments were obtained from virus RNA by reverse transcription-PCR amplification and were subsequently cloned into pEGFP-C1 to produce plasmids pEGFP-PV2C and pEGFP-CA2C.

**Yeast Two-hybrid Screening**—After verifying that the bait plasmids were expressed in the AH109 yeast strain and did not activate the reporter gene, the AH109 yeast strain was transformed with pGBK7-EV71 2C1 or 2C2 to screen an oligo(dT)-primed human fetal brain cDNA library fused to the Gal4-activating domain vector (pACT2; Clontech). The positive clones were selected on low stringency plates lacking tryptophan (Trp) and leucine (Leu). After 3–4 days of growth, the Trp+ Leu+ transformants were replica-plated to high stringency plates (lacking tryptophan, leucine, adenine, and histidine) and were incubated until colonies appeared.

**Antibodies**—Rabbit polyclonal antibodies against RTN3 were raised against His-tagged fusion proteins of full-length human RTN3, and rat polyclonal antibodies against RTN3 were raised against GST-tagged fusion proteins of full-length human RTN3. Rabbit polyclonal antibodies against enterovirus 71 3A and 2C proteins were prepared using His tag fusions to the full-length 3A and 2C1 proteins, respectively. Monoclonal antibodies against His tag and EV71 were from Serotec (Oxford, UK) and Chemicon (Temecula, CA), respectively, and rabbit polyclonal antibody against green fluorescent protein (GFP) was from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). The monoclonal antibody against c-Myc was clone 9E10. Antisera to dsRNA was used to characterize the replication complex. Poly(I-C) (Sigma) was used as a source of dsRNA, and the polyclonal antibody was prepared as described (26, 27). Briefly, methylated bovine serum albumin (Calbiochem) was mixed with an equal amount of poly(I-C) dissolved in SSC buffer (150 mM NaCl and 15 mM sodium citrate, pH 7.2), and the resultant insoluble mixture was emulsified with one part of Freund’s adjuvant. The mixture was then injected subcutaneously into Sprague-Dawley rats (BioLASCO Taiwan Co., Ltd.). The animal experiments were conducted in conditions based on the
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Chang Gung University Guiding Principles on Animal Experiment. The titer and specificities of the antibodies to poly(I-C) and viral dsRNA were characterized by an enzyme-linked immunosorbent assay and immunofluorescence microscopy (data not shown; supplemental Fig. 2).

Detergent Treatment of Virus-infected P15 Fraction—Vero cells infected with EV71 were scraped into phosphate-buffered saline (PBS) and collected by low speed centrifugation. A cytoplasmic extract was prepared by cell cracker (EMBL) homogenization in PBS containing protease inhibitors and then centrifuged twice at 1,000 × g for 10 min at 4 °C to sediment unbroken cells, nuclei, and large sheets of plasma membrane. The supernatants were centrifuged at 15,000 × g for 20 min at 4 °C to separate membrane (P15) and supernatant fractions (S15). The P15 membrane fraction was treated with 0.1 mM Na2CO3 or 1% Triton X-100 in PBS at 37 °C for 1 h and then centrifuged at 15,000 × g for 20 min. The samples were resuspended in SDS sample buffer and analyzed using 12% SDS-PAGE.

Coimmunoprecipitation Assays—HEK-293T cells (5 × 106) were transfected by electroporation at 250 V and 1000 microfarads. Cells were transfected with 5 μg of various plasmids and harvested for further assays at 24 h after transfection. Transfected cells were washed twice with PBS and scraped into lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA) with freshly added protease inhibitors. After incubation for 30 min on ice and a brief sonication, the lysate was centrifuged at 15,000 × g for 5 min at 4 °C to remove the insoluble cell debris. Equal amounts (500 μg) of lysates were incubated overnight with 1 μg of antibody at 4 °C with continuous rocking. Mouse serum as an irrelevant IgG (0.5 μl) was used as a control. After incubation with protein G beads for 3 h at 4 °C with continuous rocking, beads were washed thoroughly with lysis buffer; the sample buffer was added and further incubated for 30 min at 37 °C. The samples were resolved by 12% SDS-PAGE, transferred onto polyvinylidene difluoride paper, and analyzed by Western blotting.

Immunofluorescence Microscopy—Cell monolayers (1.5 × 105) were plated onto coverslips 1 day before transfection or infection. The cells were transfected with various constructs using Lipofectamine 2000 (Invitrogen). Transfected cells were fixed in 4% paraformaldehyde for 20 min at room temperature and then washed twice with 25 mM glycine in PBS and once with PBS. To detect the presence of dsRNA, the infected cells were first fixed in cold acetone at 8 h p.i. Nonspecific binding was blocked by incubating the cells with a blocking solution (1% gelatin, 2% bovine serum albumin, and 0.02% saponin in PBS) for 1 h at room temperature. Direct immunofluorescence was performed by incubating the cells with primary antibodies in blocking solution for 1 h at room temperature. Washes were followed by incubation with secondary antibodies diluted with blocking solution for 1 h at room temperature. Fluorescence was then evaluated using a Leica laser confocal microscope (model SP2).

Reticulon 3 Knockdown and Retransformation—The siRNA precursor of RTN3 in vector pSUPER.retro (Oligoengine, Seattle, WA) and the vector control were transfected stably into HeLa cells. Transduced subpopulations were selected using 0.5 μg/ml puromycin. The knockdown siRNA sequence is listed below with its target 19-bp stem loop sequence underlined. The sense strand was 5′-GAT CCC GGC TGT ACA GAA GTC AGA AGT TCA AGA GA CTT GGA TT A-3′, and the antisense strand was 5′-AGC TTA AAA AGC TGT ACA GAA GTC AGA AGT CTC TTG AAC TTC TGA CTT CTT TAC AGC GGG-3′. For the reintroduction of nondegradable RTN3 into siRTN3 cells, two nucleotide mutations, G126A and G129A, were introduced in the region of RTN3 (accession number AF059524) that was complementary to the siRNA, using site-directed mutagenesis. The RTN3 mutagenic primers were as follows. The N-terminal forward primer was 5′-ATG GCG GAG CCG TCG GCG GCC ACT-3′, and the reverse primer was 5′-GCG GTG CAT GAC GTG ATT TTC TTC-3′. The C-terminal forward primer was 5′-GAA AAT CAG ATC GTG CAC GCC ACA GGA GGA ACT ACA GCT CTT-3′, and the reverse primer was 5′-TTG GCC CAT TTT TTG GGC GAT TCC-3′ (substituted nucleotides are underlined). Both the N- and the C-terminal fragments of RTN3 were amplified and annealed for extension by PCR using pfu polymerase. The products were cloned into pcDNA3.1-Myc-His, and the resulting plasmid was transfected into the siRTN3 cell line to generate a rescue cell line termed 2A3 under selection of 0.5 μg/ml puromycin and 1 mg/ml G418.

GST Pulldown Assay—EV71 2C1 was cloned into the pGEX vector (Amersham Biosciences). Full-length human RTN3 was cloned into the hexahistidine-tagged fusion protein vector (pET32; Novagen, Madison, WI). Fusion proteins were prepared and further purified according to the manufacturer’s instructions. GST fusion proteins immobilized on glutathione-Sepharose beads were incubated with His-tagged RTN3 in TBS buffer (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.1% Triton X-100, and protease inhibitors) for 3 h at 4 °C. Beads were washed three times with TBS buffer, and bound proteins were eluted with glutathione elution buffer and analyzed by Western blotting.

Site-directed Mutagenesis—Site-directed mutagenesis of the N-terminal amphipathic domain of EV71 2C was designed according to Paul et al. (17). Synthetic forward primers containing the site-specific substitution in the N-terminal coding sequence were used to generate three separate mutants: pEV2C1 D10VE19V-GFP, 5′-TTA AAG AAG TT C AAC GTT GCG GCC GAT GCC GCG GCC GGG CTT GTG TCC-3′; pEV2C1 I125K-GFP, 5′-TTA AAG AAG TT C AAC GTT GCG GCC GAT GCC GCG GCC GGG CTT GTG TCC-3′; pEV2C1 K165K-GFP, 5′-TTA AAG AAG TT C AAC GTT GCG GCC GAT GCC GCG GCC GGG CTT GTG TCC-3′. The amino acid changes have been underlined; these fragments were annealed and cloned into pcDNA3.1-Myc-His.

Quantitative RT-PCR—Vector control and siRTN3 cells were infected with EV71 at an MOI of 5, and total RNA was extracted from cells at the indicated times using TRIzol (Invitrogen). The first strand of cDNA was synthesized using the Moloney murine leukemia virus reverse transcriptase for RT-PCR (Invitrogen). Real-time quantitative RT-PCR was performed in a 20-μl reaction mixture containing 50 mM forward
and reverse primers, 1× SYBR green master mix (Protech Technology Enterprise), and various amounts of template. Fluorescence emitted by SYBR green was detected on line by the ABI PRISM 7000 sequence detection system (Applied Biosystems). To quantify the changes in gene expression, the ∆Ct method was used to calculate relative -fold changes normalized against the 18 S gene, as described in Ref. 44. The Ct is defined as the cycle at which fluorescence is determined to be significantly greater than background. The ratio of vRNA to internal control was normalized to the control level at 0 h p.i., arbitrarily set to 1.

Cell Death Analysis—For EV71-induced cell death analysis, mock- and virus-infected cells at an MOI of 0.5 were stained with propidium iodide (Sigma) at 60 h p.i. and measured by flow cytometry (FACScan; BD Biosciences). CellQuest Pro version 4.0 was used for data analysis.

RESULTS

The EV71 2C Protein Is Associated with Host Membranes and the Replication Complex—EV71 2C is one of the most highly conserved proteins among all picornaviruses (28, 29). It shares 65% identity with that of the closely related PV, which binds to and rearranges mammalian membranes to form the replication complex. To investigate whether this association with mammalian membranes is also seen with the EV71 2C protein, we applied solubilization studies using buffers that allowed discrimination between peripheral and integral membrane proteins. Vero cells infected with EV71 were fractionated differentially and analyzed by immunoblotting using an anti-2C antibody (Fig. 1A). Less protein was found in the PBS-soluble cytosol fraction (S15 fraction) than in the pellet fraction (P15). P15 was treated with various conditions, including detergent (1% Triton X-100) or high pH. Under alkaline conditions (0.1 M Na2CO3, pH 11.5), most of 2C was insoluble. However, protein 2C was completely soluble in PBS containing 1% Triton X-100. From this solubility pattern and following the definition of Fujiki et al. (30), we conclude that the EV71 2C protein is indeed membrane-associated, as has previously been observed for the 2C protein of PV.

A universal feature of positive-stranded RNA viruses is the involvement of host intracellular membranes in RNA replication complex formation and function. Thus, it would be interesting to determine whether this membranous structure associated with EV71 2C is actually the viral RNA replication complex. Previous studies have demonstrated amply that the replication complex contains replicative viral dsRNA and markers of the ER. Here we used confocal microscopy to localize the replication complex using anti-dsRNA antibodies. To localize the ER, we transfected a plasmid expressing a fluorescent protein targeted to its lumen (ER-DsRed). HeLa cells were cotransfected with 2C1-GFP and ER-DsRed (c). Cells were fixed in cold acetone at 8 h p.i. and labeled with antibodies against 2C (a and b) or dsRNA (b) as indicated and then processed for fluorescent confocal microscopy. Overlay images are of the staining pattern for 2C with ER-DsRed (a) or dsRNA (b) or 2C1-GFP and DsRed (c).

Identification of Reticulon 3 as a Binding Partner for the EV71 Protein 2C by Yeast Two-hybrid Assay—To clarify the viral replication of EV71, we searched for potential partners interacting with 2C using the yeast two-hybrid approach. As a bait, we used a vector containing the N-terminal domain of 2C (aa 5–124, designated 2C2) to screen a human fetal brain cDNA library. We analyzed 1.3 × 107 library clones; 28 positive colonies were

into HeLa cells. The subcellular distribution of 2C1 was similar to the native 2C protein in that it appeared filamentous and perinuclear and colocalized with the marker for ER (Fig. 1B, panel c). Subsequently, we performed double labeling of HeLa cells with anti-dsRNA and 2C antibodies after EV71 infection. Fig. 1B (panel b) demonstrates the colocalization of fluorescent foci for dsRNA and the 2C protein of EV71. From this membrane association and morphological data, the function of the EV71 2C protein appears to be similar to its PV counterpart in that it is associated with membranous structures and may be involved in the formation or maintenance of the vesicle-associated replication complex (9).

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A. GST 2C1-GST RTN3-his

B. GFP 2C1-GFP 2C2-GFP

C. RTN1 RTN2 RTN4

D. a. RTN3 b. 2C c. overlay d. RTN3 e. dsRNA f. overlay

FIGURE 2. Analysis of the interactions between EV71 2C protein and RTN3. A, the left panel shows a Coomassie Blue-stained gel of purified proteins (2 μg each), and the right panel is the result of the GST pulldown of RTN3 with 2C1-GST or GST alone. One microgram of 2C1-GST or GST immobilized on glutathione-Sepharose beads was incubated with 1 μg of His-tagged RTN3 protein. The bound proteins were eluted with glutathione after extensive washing and were subjected to Western blotting (WB) using anti-His or anti-GST antibodies. The results presented here represent three or more independent experiments. The 2C1-GST complex tends to degrade, as shown in the figure. B, coimmunoprecipitation analysis of N-terminal fragments of 2C protein. HEK-293T cells were cotransfected with RTN3 in pcDNA3.1-Myc-His and either GFP vector or N-terminal enterovirus 71 2C proteins (2C1-GFP and 2C2-GFP) in a pEFP vector. After 24 h, transfected HEK-293T cells were lysed under non-denaturing conditions, and the cleared lysates were subjected to immunoprecipitation using an anti-His tag antibody or irrelevant mouse IgG as a control. Reactions were analyzed using 12% SDS-PAGE and immunoblotted using anti-GFP or anti-Myc antibodies. Input, 10% input of cell lysate for immunoprecipitation reaction. The arrowhead indicates antibody light chain from precipitating antibodies, which cross-react on Western blots.

C, interactions of reticulon proteins with 2C1. The pcDNA3.1-Myc-His vectors containing the RHD of RTNs 1, 2, and 4 were cotransfected into HEK-293T cells with 2C1-GFP. Cellular extracts of transfected cells were immunoprecipitated with anti-His antibody. The immunocomplexes were analyzed by confocal microscopy. The yellow fluorescent foci indicate the extent of colocalization.

identified, based on their ability to activate all of the reporters present in yeast. One colony was identified as reticulon 3, and one was identified as reticulon 1C. In another yeast two-hybrid screening experiment, we used 2C1 (aa 5–43) as the bait. Ten of 14 2C1-positive clones were identified as RTN1C. This increased our confidence that there must be interaction between 2C and members of the RTN family. Furthermore, both RTN1C and RTN3 share 60% identity at the protein level in their RHDs. Therefore, the predicted membrane/RNA binding domain of 2C is also involved in RTN binding. Both of the RTN clones identified by our yeast two-hybrid screening contain only the RHD, so this is likely to be involved in the interaction between 2C and the RTN proteins. Interestingly, in RT-PCR experiments, RTN1C was present in neuronal cells, such as PC12 and IMR32, but was absent in nonneuronal cells, such as Vero, COS1, HEK-293T, and HeLa (data not shown); this is in accordance with previous findings (18). Alternatively, all of these cell lines were positive for RTN3 (data not shown). Because of the wider expression of RTN3, we chose to focus on RTN3 for most experiments.

In Vitro and in Vivo Association of the EV71 2C Protein with RTN3—To further confirm the interaction between EV71 2C1 and RTN3, we performed an in vitro binding assay using recombinant fusion proteins. Full-length RTN3 and EV71 2C1 were cloned into a hexahistidine- and a GST-tagged fusion vector, respectively. GST-2C1 and GST were bound to glutathione-Sepharose beads, and His-RTN3 protein was added and incubated with gentle shaking. The bound proteins were eluted and electrophoresed on a polyacrylamide gel, and His-RTN3 was detected by Western blotting using a monoclonal anti-His antibody. In these experiments, RTN3 bound only to GST-2C1 but not to GST alone, demonstrating that the interaction between EV71 2C1 and RTN3 was direct and specific (Fig. 2A). The interaction between EV71 2C and RTN3 was also confirmed by coimmunoprecipitation experiments (Fig. 2B). HEK-293T cells were transfected with expression vectors encoding GFP-2C1, GFP-2C2, and GFP control vector in combination with epitope-tagged RTN3 (RTN3-Myc-His). The transfected cells were solubilized in lysis buffer, and anti-His antibodies were used to pull down the immunocomplex. Cell lysates and the immunocomplex samples eluted from the protein G beads were subjected to SDS-PAGE and immunoblotted using anti-GFP and anti-Myc antibodies. When GFP-2C1 or GFP-2C2 was coexpressed with RTN3 tagged with Myc-His, single bands cor-

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responding to the recombinant 2C1 or 2C2 proteins were detected in the bead eluates (Fig. 2B, lanes 5 and 8). These bands were not detected when control IgG was used (lanes 6 and 9) or when a control vector encoding only GFP was used (lanes 1–3), again confirming the specificity of the interaction. The full-length EV71 2C was able to interact with RTN3 with similar efficiency (see Fig. 3).

To confirm whether RTN3 interacts with EV71 (nonrecombinant) 2C protein in infected cells, we transfected HEK-293T cells with Myc-His-tagged RTN3 overnight followed by infection with EV71. The lysates were prepared for coimmunoprecipitation assay with anti-His antibodies, and the immunocomplexes were examined by Western blotting. The anti-His but not the control (mouse IgG) antibodies clearly immunoprecipitated a band corresponding to the expected size of 2C on a blot in the immunocomplex (data not shown).

The EV71 2C Protein Interacts with the RHD of All Known RTN Proteins—Each member of the reticulin family shares a conserved C-terminal RHD and has multiple spliced variants (18). The RHD features two potential transmembrane domains and a 66-amino acid hydrophilic loop, designated Nogo66 for RTN4/Nogo. Because both RTN3 and RTN1C were positive clones in the yeast two-hybrid assay, we speculated that EV71 2C might bind all of the reticulin proteins. To test this hypothesis, we cloned the RHD of the other two reticulin members, RTN2 and RTN4, from the human fetal brain cDNA library. Each of these reticulons was subcloned in frame with a Myc-His epitope tag at its C terminus into a mammalian expression vector, which produced the expected protein products after transfection into HEK-293T cells. To examine the binding of each reticulin to EV71 2C, we prepared lysates from the cotransfected cells and used a monoclonal antibody to His tag or mouse IgG for coimmunoprecipitation. The EV71 2C protein coimmunoprecipitated with RTN1C, RTN2, and RTN4 (Fig. 2).

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Colocalization of the Virally Encoded 2C Protein with Endogenously Expressed RTN3 in HeLa Cells—To verify whether endogenous RTN3 could colocalize morphologically with the 2C protein or the replication complex, HeLa cells were infected with EV71 at a MOI of 5, and the cells were subjected to immunofluorescent staining with anti-RTN3 or mouse IgG for coimmunoprecipitation. The EV71 2C protein coimmunoprecipitated with RTN1C, RTN2, and RTN4 (Fig. 2). Such a band was not detected when control IgG was used. Thus, EV71 2C specifically interacts in vivo with the RHD of all known reticulin proteins. The RHD of RTN3 was the shortest domain we were able to express and test.

Coimmunoprecipitation of Enteroviral 2C Proteins with Exogenously Expressed RTN3 in HeLa Cells—Our results indicated that the 2C protein of EV71 interacts specifically with the host-encoded RTN3 protein. In addition, we demonstrated that the 2C protein of EV71 might also interact with all reticulin proteins via their conserved RHD. We were thus interested in investigating whether RTN3 could interact with the 2C proteins of other enteroviruses.

Because the nonstructural protein 2C is highly conserved among different enteroviruses, we tested whether RTN3 could interact with the 2C proteins of PV and CA16 (Fig. 3). The 2C1 region of EV71 shares 92 and 78% identity with CA16 and PV, respectively. Full-length 2C proteins from EV71, CA16, or PV were fused to pEGFP and cotransfected with Myc-His-tagged RTN3 into HEK-293T cells. Anti-His antibodies or control antibodies were used on cell lysates in this immunoprecipitation experiment. The interaction between all three 2C proteins and RTN3 was observed when we used anti-His antibodies (Fig. 3, lanes 2, 5, and 8) but not in the control lanes with mouse IgG (Fig. 3, lanes 3, 6, and 9). Thus, RTN3 may be involved in the viral replication or pathogenesis of multiple enteroviruses.

Site-directed Mutagenesis of 2C1 Defines Isoleucine 25 as Essential for RTN3 Interaction—To determine the amino acids involved in the interaction of RTN3 with EV71 2C, we introduced mutations in the vector containing protein 2C1. PV 2C is the most conserved polypeptide among picornaviruses with respect to amino acid sequence; the 2C1 region (aa 5–43) is particularly well conserved and possesses an amphipathic helix (Fig. 4A). Paul et al. (17) have made several mutations in the PV protein 2C amphiphatic region. We created the same mutations in the EV71 2C1 region. In Fig. 4A, in the first mutant (EV71 2C1 D10VE19V), we have selected two conserved acidic amino acids for mutagenesis that are located at the boundary of the hydrophobic and hydrophilic half. These two acidic amino acids were replaced by two hydrophobic valines. The second mutant (EV71 2C1 I25K) contained an isoleucine-to-lysine conversion in the hydrophobic half, and in the third mutant (EV71 2C1 K16TK24T), two lysines were converted to threonines in the hydrophilic half. These mutants were cloned into the mammalian expression vector pEGFP and were individually cotransfected with pcDNA3.1-RTN3-Myc-His into HEK-293T cells for coimmunoprecipitation assays. When either 2C1
D10VE19V or 2C1 K16TK24T was coexpressed with the recombinant 2C1 mutant protein were detected in the bead eluate (Fig. 4B, lanes 2 and 5). Such a band was not detected when the 2C1 I25K vector was cotransfected with Myc-His-tagged RTN3 and a GFP fusion product of EV71 2C1 D10VE19V (lanes 1–3), EV71 2C1 K16TK24T (lanes 4–6), or EV71 2C1 I25K (lanes 7–9). Transfected HEK-293T cells were lysed under non-denaturing conditions, and the cleared lysates were subjected to immunoprecipitation using control mouse IgG or an anti-His antibody. Reactions were analyzed on 12% SDS-PAGE and immunoblotting (WB) using anti-GFP or anti-Myc antibodies. Input, 10% of total cell lysate used in the immunoprecipitation assay. The arrowheads indicate antibody light chain. The arrows indicate the various mutants of 2C1 fused to GFP (top) or the Myc tagged RTN3 protein (bottom).

FIGURE 4. A, helical wheel diagram of the putative amphipathic helical structure (amino acid residues 18–35) of the 2C1 region of EV71. The amino acid residues of the hydrophobic side are boxed, and the hydrophilic amino acids are stretched on the other side of the helix. This diagram is redrawn using the HelicalWheel program of SeqWeb version 2.0. B, interaction of RTN3 with 2C1 variants. HEK-293T cells were cotransfected with Myc-His-tagged RTN3 and a GFP fusion product of EV71 2C1 D10VE19V (lanes 1–3), EV71 2C1 K16TK24T (lanes 4–6), or EV71 2C1 I25K (lanes 7–9). Transfected HEK-293T cells were lysed under non-denaturing conditions, and the cleared lysates were subjected to immunoprecipitation using control mouse IgG or an anti-His antibody. Reactions were analyzed on 12% SDS-PAGE and immunoblotting (WB) using anti-GFP or anti-Myc antibodies. Input, 10% of total cell lysate used in the immunoprecipitation assay. The arrowheads indicate antibody light chain. The arrows indicate the various mutants of 2C1 fused to GFP (top) or the Myc tagged RTN3 protein (bottom).

RTN3 Is Required and Sufficient for Immediate Early Virus Replication and Translation—Our site-directed mutagenesis results demonstrated that RTN3 may be involved in viral replication. To further assess the role of RTN3 in this, we used RNA interference approaches to knockdown RTN3 mRNA in HeLa cells. A knockdown cell line called siRTN3 was established. This did not produce morphological abnormalities or affect the viability of siRTN3 cells (data not shown), but RTN3 protein levels were reduced dramatically (Fig. 5A, lane 3). The vector control clonal cell line demonstrated no reduction in RTN3 levels compared with the parental HeLa cell (lane 1 versus lane 2). The siRNA technique sometimes introduces nonspecific off-target gene knockdowns (33, 34). This knowledge prompted us to reintroduce the RTN3 gene into the knockdown cell line. Taking advantage of the degenerate genetic code, a cell line called 2A3 was established, in which nondegradable RTN3 in pcDNA3.1-Myc-His was reintroduced into the knockdown cell line. Two nucleotide point mutations (G126A and G129A) were introduced by site-directed mutagenesis in the region of RTN3 complementary to the siRNA by site-directed mutagenesis. Transfected cells were obtained with puromycin selection, except that the 2A3 cells were selected with an additional 1 mg/ml of G418. Fifty-microgram aliquots of extracts were probed with the anti-RTN3 antibody by Western blot analysis (top). Membranes were also probed with an antibody against α-actin, used to control the loading of lanes (bottom). B, time course analysis of viral protein expression in siRNA and rescued cell lines. The cells were infected with EV71 at an MOI of 5. The cells were harvested at the indicated time points, and 50-μg aliquots of each cell lysate were used for Western blot analysis using antibodies against 2C, 3A, and RTN3. The mock control lacked viral infection. The arrowhead indicates precursor protein 3AB.
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FIGURE 6. Viral protein and dsRNA synthesis in RTN3 knockdown and rescued cells. A, reduced viral protein and dsRNA synthesis in RTN3 knockdown cells. HeLa parental cells (a) and stable cell lines of the vector (b), siRTN3 (c and d), and 2A3 (e) were first infected with EV71 at an MOI of 5, and the cells were harvested at 8 h p.i. for confocal microscopy using antibodies as indicated. Phase-contrast images are shown in c and d. The results shown are representative of at least three experiments. B, viral infectivity is modulated by RTN3. These four different cell lines were treated as above and were then subjected to immunofluorescence staining with anti-EV71 antibodies; the cells with immunofluorescence foci of EV71 were defined as infected cells. The infectivity rate was calculated as infected cells as a percentage of 100 randomly selected cells per field; three fields were scored. Values are means ± S.E. from two independent experiments. *, p < 0.005 compared with vector control. C, determination of viral RNA in RTN3 knockdown cells by quantitative RT-PCR. Vector control and siRTN3 cells were infected with EV71 at an MOI of 5, and total RNA was extracted from cells at the indicated times. To quantify the changes in gene expression, the ΔCt method was used to calculate relative -fold changes normalized against the 18S gene. The experiment was performed three or more times. The ratio of vRNA to internal control was normalized to the control level at 0 h p.i., arbitrarily set to 1.

was also demonstrated by immunofluorescent staining, as shown in Fig. 6A. The levels of 2C protein in parental cells and vector control cells were similar (Fig. 6A, a and b), consistent with the data shown in Fig. 5B. In the siRNA knockdown cells (siRTN3), the RTN3 level fell, and production of the 2C protein was ablated (Fig. 6A, panel c). However, the RTN3 rescue cell line 2A3 had strong synthesis of viral protein and RNA (Fig. 6A, panel e). Synthesis of replicative dsRNA was also markedly reduced in the siRTN3 cell line, as detected by confocal microscopy (Fig. 6A, panel d). The transmitted light images are to demonstrate that the loss of immunofluorescent foci was not caused by a lack of cells. By contrast, the parental cells and vector control cells displayed similar dsRNA synthesis levels (panels a and b). These morphological data demonstrated a global reduction of viral protein and dsRNA synthesis. We have also analyzed the viral RNA synthesis directly by quantitative RT-PCR, and the viral RNA synthesis was significantly reduced in siRTN3 cells (Fig. 6C). We then calculated the infectivity of these cells. This is defined by the ratio of viral protein-positive cells detected with anti-EV71 antibody by confocal microscopy. These results were quantified by counting the ratio of infected cells among randomly selected cells (Fig. 6B). The controls of wild type or vector-transfected cells showed infection rates of about 24–28%, but fewer than 5% of RTN3 knockdown cells were infected. In 2A3 cells, the infection rate increased to the wild type level of 27%. This suggests that RTN3 is necessary and sufficient for viral infection and demonstrates that our design for RNA interference was specific, because the reintroduction of the gene for RTN3 rescued the efficiency of viral infection. Taken together, these data are consistent with the idea that RTN3 functions as a modulator of viral replication activity in these host cells. The siRTN3 and control cells were also subjected to synchronized infection with virus to establish the kinetics of viral propagation (Fig. 7A). The data showed that the decrease in -fold virus titer in RTN3 knockdown cells compared with the vector control cells started from about 1.5-fold (at 11 h p.i.) to 4-fold (at 7 h p.i.) (Fig. 7A), accompanied by markedly reduced viral translation and level of dsRNA (Figs. 5 and 6). Such a reduction of viral proteins should delay or prevent EV71-induced apoptosis (cytopath). Therefore, enterovirus 71-induced apoptosis was analyzed quantitatively by fluorescence-activated cell sorting (Fig. 7B). When enterovirus 71 was added, the vector control and wild type cells showed a prominent new peak representing the sub-G1 phase characteristic of cells undergoing apoptosis. The enterovirus 71-induced sub-G1 phase had no change, if any, in the siRTN3 cells. Thus, knockdown of RTN3 inhibited enterovirus 71-induced apoptosis in HeLa cells. The siRTN3 cells also displayed a reduced cytopathic effect cytologically.
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A. Single-cycle growth analysis of virus in RTN3-depleting cells. The siRTN3 and vector cells were infected at a MOI of 5 and incubated at 37 °C. At 0, 3, 5, 7, 9, or 11 h p.i., viruses were released from the infected cells by three cycles of freezing and thawing. Viral titers were determined by plaque assay, and the ratio of viral yield was normalized to the control level at 0 h p.i., set arbitrarily to 1. Values are means from two independent experiments. B. Repression of EV71-induced cell death by RTN3 siRNA. Stable clones of HeLa transfected with empty vector or RTN3 siRNA were infected with EV71 at a MOI of 0.5. At 60 h p.i., adherent and floating cells were harvested and fixed with ethanol, and cell cycle progression was analyzed by flow cytometry. DNA content was quantified by staining the cells with propidium iodide (PI). The numbers indicate the percentage of cells in the sub-G1 phase of the cell cycle after infection.

DISCUSSION

Here we showed that EV71 could subvert cellular protein RTN3 for viral infection. RTN3 directly interacted with EV71 protein 2C, and EV71 infection in cells with an siRNA knockdown of RTN3 lost the ability to synthesize viral RNA and protein (Figs. 5 and 6). The reduced viral progeny numbers and apoptosis resulted from defective synthesis of viral protein and RNA in EV71-infected siRTN3 cells, as demonstrated by plaque assays and fluorescence-activated cell sorting assays, respectively (Fig. 7). Additionally, we showed that the ectopic expression of a nondegradable form of RTN3 in siRTN3 knockdown cells could rescue the infectivity caused by EV71. Therefore, we suggest that RTN3 plays a central role in EV71 replication.

PV has been widely used as a model system for the studies of positive-stranded RNA virus replication complex biogenesis. Infection with PV results in a range of membrane morphologies, many of which involve complex membrane rearrangements to form replication complex-associated vesicles. These vesicles are derived from organelles involved in the secretory pathway, mainly from ER but also possibly from the Golgi complex. Several reports indicate that protein 2C can bind to cell membranes and thus induce vesicle formation through its N-terminal amphipathic region in the absence of other viral proteins (6–8, 36). We engineered amino acids in this putative amphipathic region near the N terminus of 2C and tested their effects on the interaction with RTN3. An I25K substitution was created to interrupt the continuous stretch of hydrophobic amino acids on the nonpolar face, so that the change would in effect abolish the entire amphipathic nature of the helix and its ability to bind membrane. This mutant in PV 2C causes a reduction in negative strand RNA synthesis (17). When the EV71 mutant plasmid was cotransfected for immunoprecipitation assays, no interaction between 2C and RTN3 was detected. We also observed that this mutant has lower levels of EV71-induced CPE than the wild type (data not shown). Thus, we suggest that the reduced viral RNA synthesis in this I25K mutant observed by Paul et al. (17) was caused by the loss of interaction between 2C and RTN3, thus decreasing RNA synthesis.

Alternative splicing generates at least five isoforms of RTN3, all of which contain a conserved C-terminal 188-aa RHD but with divergent N-terminal regions. Some RTN3 isoforms have immense N-terminal regions of up to 800 aa (e.g. human RTN3A3b and -4b have 1013 and 1032 amino acids, respectively) (25). At present, little is known about the functions of
these bulky N-terminal domains. The siRNA duplex reported here was designed to target the RHD and thus, theoretically, to knock down all of the isoforms of the RTN3 family. These RTN3-depleting cells were found to be viable, thus demonstrating that RTN3 is not an essential gene for cell survival. However, the reintroduction of a relatively small isoform of only 236 amino acids (RTN3A1, as defined by Di Scala ever, the reintroduction of a relatively small isoform of only 236 amino acids (RTN3A1, as defined by Di Scala et al. (25)) rescued viral infectivity (Figs. 5 and 6). Thus, it is the RHD rather than the bulky N-terminal domain that is required for viral replication. However, the N-terminal domain might play a role in regulating the interaction with 2C protein. We have shown that all members of the reticulon family of proteins, with the full-length protein or just the conserved RHD, bind to 2C (Fig. 2C and supplemental Fig. 1). The efficiency of the interaction of four RHDs with 2C is similar, but the efficiency of the interaction of the full-length RTN protein with 2C is different, suggesting that the N-terminal domain regulates the binding and modulates the infectivity of EV71 and possibly other enteroviruses.

Vesicular structures generated in the PV-infected cells appear to play a crucial role as compartments where synthesis of viral RNA takes place. These structures are derived sequentially from membranes of different cellular organelles. At early times following infection, PV-induced vesicles are probably formed in the ER (3, 5, 37), whereas at later times, membranes from other cellular organelles, including lysosomes and the Golgi apparatus, may also contribute to vesicle formation (4, 5).

RTN3 is an ER-associated protein, and its reduced expression in knockdown cells may affect the budding of replication complex vesicles manufactured from the ER compartment membrane (Fig. 6). An RTN family protein interacts with soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (21) and is also involved in membrane trafficking between the endoplasmic reticulum and Golgi (22). SNAREs comprise three families of proteins: a synaptosomal-associated protein of 25 kDa (SNAP-25) and its related isoforms, the syntaxins, and vesicle-associated membrane proteins. SNARE proteins are important for docking and fusion of the transport vesicles to target membranes (see review in Ref. 38). Cleavage of vesicle-associated membrane protein caused defective synaptic vesicle biogenesis in PC12 cells (39). The SNARE protein also participates in COPII vesicle formation (40). PV replication complex-associated vesicles are formed in the ER by the cellular COPII budding mechanism and are similar to the vesicles of the anterograde membrane transport pathway (3). COPII coat proteins are required for direct capture of cargo and SNARE proteins into transport vesicles from the ER. Such interaction of RTN proteins with the SNARE proteins is thus in accordance with the finding that PV replication complex-associated vesicles are associated with COPII from which the vesicles are budded from the donor ER membrane (3). The protein 2C has two regions involved in RNA binding: one in the N-terminal amphipathic region located between amino acids 21 and 45. Sequence analysis shows that 2C does not contain any hydrophobic transmembrane domain, and it may interact with host proteins (7, 16). We speculate that RTN3 is the potential host protein that interacts with 2C for the process of vesiculation and thus may be involved in the transport of 2C along with viral RNA to form the replication complex via a COPII-de-
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