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D. P. Gladue
USDA-ARS, Douglas.Gladue@ars.usda.gov

V. O' Donnell
USDA-ARS

R. Baker-Branstetter
USDA-ARS

L. G. Holinka
USDA-ARS

J. M. Pacheco
USDA-ARS

See next page for additional authors

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Foot-and-Mouth Disease Virus Modulates Cellular Vimentin for Virus Survival

D. P. Gladue,[a] V. O'Donnell,[a] R. Baker-Branstetter,[a] L. G. Holinka,[a] J. M. Pacheco,[a] I. Fernández Sainz,[a] Z. Lu,[b] X. Ambroggio,[c] L. Rodriguez,[a] M. V. Borca[a]

Plum Island Animal Disease Center, ARS, USDA, Greenport, New York, USA[a]; Plum Island Animal Disease Center, DHS, Greenport, New York, USA[b]; Bioinformatics and Computational Biosciences Branch, NIAID, NIH, Bethesda, Maryland, USA[c]

Foot-and-mouth disease virus (FMDV), the causative agent of foot-and-mouth disease, is an Aphthovirus within the *Picornaviridae* family. During infection with FMDV, several host cell membrane rearrangements occur to form sites of viral replication. FMDV protein 2C is part of the replication complex and thought to have multiple roles during virus replication. To better understand the role of 2C in the process of virus replication, we have been using a yeast two-hybrid approach to identify host proteins that interact with 2C. We recently reported that cellular Beclin1 is a natural ligand of 2C and that it is involved in the autophagy pathway, which was shown to be important for FMDV replication. Here, we report that cellular vimentin is also a specific host binding partner for 2C. The 2C-vimentin interaction was further confirmed by coimmunoprecipitation and immunofluorescence staining to occur in FMDV-infected cells. It was shown that upon infection a vimentin structure forms around 2C and that this structure is later resolved or disappears. Interestingly, overexpression of vimentin had no effect on virus replication; however, overexpression of a truncated dominant-negative form of vimentin resulted in a significant decrease in viral yield. Acrylamide, which causes disruption of vimentin filaments, also inhibited viral yield. Alanine scanning mutagenesis was used to map the specific amino acid residues in 2C critical for vimentin binding. Using reverse genetics, we identified 2C residues that are necessary for virus growth, suggesting that the interaction between FMDV 2C and cellular vimentin is essential for virus replication.

Foot-and-mouth disease (FMD), a highly contagious viral disease of cattle, pig, sheep, goats, and wild cloven-hoofed animals, is caused by foot-and-mouth disease virus (FMDV), a single-stranded positive-sense RNA virus. There are seven serotypes (A, O, C, Asia, SAT1, SAT2, and SAT3) of FMDV that do not offer cross-protection (1, 2). Four structural proteins (VP1, VP2, VP3, and VP4) comprise the infectious nonenveloped icosahedral virion. The genome has a single large open reading frame (ca. 7,000 nucleotide [nt]), which is translated to make the polyprotein which is processed by the two viral proteases Lpro and 3C and by a ribosomal skip mechanism in 2A into the polypeptide products L, P1-2A, P2 (2B and 2C), and P3 (3A, 3B1-3, 3Cpro, and 3Dpol). Further cleavage of these regions yields 14 mature virus proteins, along with several protein intermediates, that are critical for viral replication (3, 4).

During replication, FMDV causes several rearrangements of intracellular membranes, resulting in vesicular structures that contain viral proteins, which are part of the replication complex. Replication complexes have been associated with many other positive-strand RNA virus infections (5–11). FMDV has been shown to modulate the autophagosome pathway through the interaction of FMDV 2C with a central cell regulator of autophagy, Beclin1 (12). FMDV 2C, a 318-amino-acid protein, has also been shown to play a role in disruption of the Golgi-ER secretory pathway (13). However, it is possible that 2C can play multiple roles in the process of virus replication and that 2C may interact with several host cellular factors during infection. To gain insight into possible cellular factors that could interact with 2C helping to form these replication structures, we have been utilizing a yeast two-hybrid approach to identify host cell proteins that interact with 2C. We recently reported that cellular Beclin1 is a natural ligand of 2C and that it is involved in the process of autophagy which was shown to be important for FMDV replication (12, 14). We now report that cellular vimentin is also a specific binding partner for viral 2C. Vimentin is a class III intermediate filament (IF), a predominant IF in cells of the vascular endothelium. Vimentin has been shown to be associated with several cellular organelles, including autophagosomes, and to have a role in lysosomal degradation of proteins (15, 16).

Vimentin has been shown to be important during the replication cycle of various viruses. It is involved in the process of viral entry of cowpea mosaic virus (17) and Japanese encephalitis virus (18) and in the viral egress of bluetongue virus (19). It has also been implicated in the process of viral replication of vaccinia virus (20) and dengue virus (21). Although the significance is not clear, vimentin is cleaved in cells infected with human immunodeficiency viruses (22) and adenovirus type 2 (23), and its transcription significantly increases during infection with human T-cell leukemia virus type I (24). In addition, in cells infected with African swine fever virus (25) or iridovirus frog virus 3 (26), vimentin surrounds virus factories. These vimentin "cage-like" structures containing viral proteins have been shown to be important for virus survival (25). It is also possible that vimentin serves a potential protective host function, as vimentin has been implicated to be involved in the isolation and clearance of misfolded proteins in cells (15, 27). In addition, vimentin has a possible involvement in

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Address correspondence to M. V. Borca, pmanuel.borca@ars.usda.gov.

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the autophagy pathway since it plays an important role in positioning autophagosomes, lysosomes, and the Golgi complex within the cell (15).

In this report we further examine the 2C-vimentin interaction identified by yeast two-hybrid analysis, confirming by coimmunoprecipitation that this interaction occurs in FMDV-infected cells. Immunofluorescence staining in FMDV-infected cells revealed transient formation of a vimentin cage structure that surrounds FMDV protein 2C. Importantly, expressing a dominant-negative (DN) form of vimentin resulted in the disruption of the physiological disposition of vimentin and in a significantly decreased ability for FMDV to replicate, suggesting that interaction with vimentin plays an important and necessary role during viral infection. In addition, the site of interaction in the 2C protein that is responsible for binding to vimentin was mapped by alanine scanning mutagenesis. Using reverse genetics it was shown that changes to amino acid residues of 2C responsible for binding vimentin are clearly detrimental for virus replication. This further supports the hypothesis that 2C binding to vimentin may play a significant and necessary role for virus replication.

MATERIALS AND METHODS

Cell lines, viruses, and plasmids. Human mammary gland epithelial cells (MCF-10A) were obtained from the American Type Culture Collection (catalogue no. CRL-10317) and maintained in a mixture of Dulbecco minimal essential medium (DMEM; Life Technologies) and F-12 Ham media (1:1; Life Technologies, Grand Island, NY) containing 5% heat-inactivated fetal bovine serum (HI-FBS, Thermo Scientific, Waltham, MA), 20 ng of epidermal growth factor (Sigma-Aldrich, St. Louis, MO)/ml, 100 ng of cholera toxin (Sigma-Aldrich)/ml, 10 μg of insulin (Sigma-Aldrich)/ml, and 500 ng of hydrocortisone (Sigma-Aldrich)/ml.

FMDV type O1 strain Campos (FMDV O1C) was derived from the vesicular fluid of an experimentally infected steer. The virus was amplified once in baby hamster kidney-21 (BHK-21) cells, and the titer determined by plaque assay on (BHK) cells using standard techniques (14).

Plasmids vimentin-HA and vimentin DN-HA were generously donated by Stewart Martin's laboratory (University of Maryland School of Medicine, Baltimore, MD). Plasmid phrGFP II-N mammalian expression vector is commercially available (Agilent Technologies, Santa Clara, CA).

For viral replication studies, MCF-10A cells were plated at a density of 10^6 per well in a six-well plate (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ). Indicated plasmids were transfected into cells using FuGene (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. After 24 h, the cells were infected with FMDV O1C at the specified multiplicity of infection (MOI) or mock infected. Virus was allowed to adsorb for 1 h, followed by an acid wash with ice-cold 145 mM NaCl–25 mM MES (morpholineethanesulfonic acid; pH 5.5) to remove residual virus particles and the addition of fresh medium containing 0.5% HI-FCS. Samples were taken at the indicated time points.

Antibodies and reagents. Monoclonal antibody (MAb) 3D10, directed against the FMDV O1 nonstructural protein 2C, was developed in the Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna, Brescia, Italy. The vimentin-specific MAb for staining and coimmunoprecipitation was from Sigma-Aldrich (V6630). The MAb specific for the hemagglutinin (HA) tag used for Western blot analyses was produced by Millipore (clone DW2). The actin-specific MAb was from Millipore (MAb1501). Acrylamide (Sigma-Aldrich) was made as a 4 M stock in water and diluted in media to the indicated concentrations. Nocodazole (Sigma-Aldrich) was made as a 40 mM stock and diluted to the indicated concentrations. Cell proliferation kit I (MTT; Roche catalog no. 11465007001) analysis was performed according to the manufacturer's instructions.

Infection and transfection of cells for immunofluorescence staining. Subconfluent monolayers of MCF-10A cells grown on 12-mm glass coverslips in 24-well tissue culture dishes were transfected with the indicated plasmids. After 24 h, they were infected with FMDV O1C at an MOI of 10 in minimum essential medium (MEM; Life Technologies, Grand Island, NY) containing 0.5% HI-FBS, 25 mM HEPES (pH 7.4), and 1% antibiotics. After the 1-h adsorption period, the supernatant was removed, and the cells rinsed with ice-cold 2-morpholinoethanesulfonic acid (MES) buffered saline (25 mM MES [pH 5.5], 145 mM NaCl) to remove unadsorbed virus. The cells were washed once with medium before fresh medium was added and then incubated at 37°C and 5% CO_2. At the indicated time points after infection, the cells were fixed with 4% paraformaldehyde (EMS, Hatfield, PA) and analyzed by immunofluorescence staining. To express HA-vimentin, HA-DN-vimentin, or green fluorescent protein (GFP) protein, monolayers of MCF-10A cells were transfected with 0.5 μg of plasmid DNA using FuGene (Roche, Mannheim, Germany), according to the manufacturer's recommendations. At 19 to 24 h posttransfection, the cells were infected as described above and then fixed with 4% paraformaldehyde (EMS) at the appropriate times.

After 15 min of fixation, the paraformaldehyde was removed, and the cells were permeabilized with 0.5% Triton X-100 for 5 min at room temperature, followed by incubation in blocking buffer (phosphate-buffered saline [PBS], 5% normal goat serum, 2% bovine serum albumin, 10 mM glycine, 0.01% Thimersal) for 1 h at room temperature. The fixed cells were then incubated with the primary antibodies overnight at 4°C. When double labeling was performed, cells were incubated with both antibodies together. After being washed three times with PBS, the cells were incubated with the appropriate secondary antibody, goat anti-rabbit immunoglobulin G (IgG) (1/400; Alexa Fluor 594 or Alexa Fluor 647; Molecular Probes), goat anti-mouse isotype-specific IgG (1/400; Alexa Fluor 488 or Alexa Fluor 594; Molecular Probes), for 1 h at room temperature. After this incubation, the coverslips were washed three times with PBS, counterstained with the nuclear stain TOPRO-iodide 642/661 (Molecular Probes) or DAPI (4′,6′-diamidino-2-phenylindole; Life Technologies, Grand Island, NY) for 5 min at room temperature, washed as before, mounted, and examined using a Nikon Eclipse 90i microscope. The data were collected utilizing appropriate prepared controls lacking the primary antibodies, as well as using anti-FMDV antibodies in uninfected cells to give the negative background levels and to determine channel crossover settings. The captured images were adjusted for contrast and brightness using Adobe Photoshop software.

Coimmunoprecipitation of FMDV 2C and vimentin. MCF-10A cells were grown to 90% confluence and then infected with a MOI of 10 or mock infected. Cells were lysed at 2.5 h postinfection using protease inhibitors (Protea Biosciences, Morgantown, WV) and radioimmunoprecipitation assay (RIPA) buffer (Teknova, Hollister, CA). For immunoprecipitation the protein lysate was incubated with protein G-beads (Sigma-Aldrich) precoupled to a MAb directed against vimentin (Sigma-Aldrich V6630). The cell lysate was incubated with anti-vimentin antibody for 2.5 h and then incubated with the antibody and beads overnight at 4°C. The beads were washed five times using RIPA buffer, and then protein elutes were collected for each sample and examined by Western blotting probing for anti-2C (3D10).

Library screening. A bovine cDNA expression library was constructed (12), where cellular proteins were expressed as GAL4-AD fusion proteins. The GAL4-based yeast two-hybrid system provides a transcriptional assay for detection of protein-protein interactions (28, 29). The bait protein, FMDV O1C 2C protein, was expressed with an N-terminus fusion to the GAL4-binding domain (BD). Full-length 2C protein (amino acids 1082 to 1399 of the FMDV polyprotein) was used for screening and for full-length mutant protein construction. Screening used FMDV 2C as bait and testing of positive clones recovered from the bovine library was done (12). The vimentin recovered from the library matched bovine vimentin (NCBI reference sequence NP_776394.2).

Western blot quantification. To quantify Western blots, we used ImageJ software provided by http://imagej.nih.gov/ij/ (30). Actin was used as a loading control. To calculate the percentage of vimentin in each sample the relative density of vimentin was divided by the relative density of actin.

Site-directed mutagenesis. Full-length pO1Ca (31) or 2C-BD was used as a template in which amino acids were substituted with alanine, introduced by site-directed mutagenesis using QuikChange XL site-directed mutagenesis (Stratagene, Cedar Creek, TX) performed according to the manufacturer's instructions (12).

Primers were designed using the manufacturer's primer design program (https://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Tool&SubPageType=ToolQCPD&PageID=15), which limited us to a maximum of seven amino acid changes and provided the rationale for deciding on the regions to be mutated.

Construction of mutant FMDV viruses. Plasmid pO1Ca or its mutant version was used as a template for RNA synthesis (12). BHK-21 cells were transfected with these synthetic RNAs by electroporation (Electrocell Manipulator 600; BTX, San Diego, CA) (31, 32). The supernatants from transfected cells were passed in LF-BK αVβ6 cells until a cytopathic effect (CPE) appeared or until a minimum of four blind passages was performed and no evidence of CPE was observed. After successive passages in these cells, virus stocks were prepared and the viral genome completely sequenced using the Prism 3730xl automated DNA sequencer (Applied Biosystems) (31).

Bioinformatic analysis of FMDV 2C. The FMDV 2C amino acid sequence was queried with BLAST (33) and HHpred (34) against the PDB and HHpred PDB70 database, respectively. The BLAST search of the 2C amino acids sequence against the PDB resulted in a match between residues 85 and 177 of 2C with residues 4 to 91 of a putative fructose transport system kinase protein from *Silicibacter* (*Silicibacter* fructokinase) with an E value of 0.17. HHpred identified two viral AAA+ ATPases, the simian virus 40 large T antigen (simian virus 40 LT) and adeno-associated virus 2 REP40 (Aav2 Rep40) protein, with E values of 2.5E−08 and 2.5E−06, respectively. For the former, the identified region of homology began at amino acid 109 of 2C, whereas for the latter, it began at amino acid 18. We used a structural alignment of the monomeric units of *Silicibacter* fructokinase (PDB ID 3C8U), SV40 LT (PDB ID 1SVM), and Aav2 Rep40 (1U0J) and the sequence alignment of 2C with *Silicibacter* fructokinase and Aav2 Rep40. These crystal structures were superposed using LSQMAN, from which a structure-based sequence alignment was extracted. Alanine scanning blocks 12 and 13 were mapped, respectively, to the exposed loop preceding helix 0 of the AAA+ domain of the SV40 LT crystal structure (residues 395 to 400) and the first half of this helix (residues 401 to 407).

RESULTS

FMDV nonstructural 2C protein interacts with the bovine host protein vimentin. Nonstructural FMDV 2C is a highly conserved (>85% identity) 318-amino-acid protein, essential for virus replication (1, 31). This high degree of conservation suggests a possible essential function during virus infection.

A yeast two-hybrid system (35) was used to identify interactions between host cellular proteins and FMDV 2C protein (12). An N-terminal fusion of the Gal4 protein DNA-binding domain (BD) with FMDV 2C protein from FMDV O1C was used as "bait". For "prey" we used a custom cDNA library that was derived from RNA extracted from FMDV-susceptible bovine tissues expressed as N-terminal fusion of the Gal4 activation domain (AD). Library screening was performed as described by Gladue et al. (35). One specific protein binding partner for 2C, vimentin (NCBI reference sequence NP_776394.2) (Fig. 1A), was selected for further study due to its potential involvement in the rearrangement of intracellular membranes (36–38) and positioning of autophagosomes (15, 39), both processes known to be important in FMDV replication (12–14).

Coimmunoprecipitation and immunofluorescence staining were used to confirm that the interaction identified using the two-hybrid system in yeast actually occurs during FMDV infection of host cells. Coimmunoprecipitation experiments were performed using cell lysates from FMDV-infected human epithelial cell line MCF-10A, an FMDV-susceptible cell line which allows for use of MAbs recognizing human proteins, and MAbs specifically recognizing FMDV 2C and vimentin. MCF-10A cells were infected with an MOI of 1 of FMDV O1C, and samples were harvested at 1.5 to 2 h postinfection (hpi), the time point when 2C is beginning to accumulate in MCF-10A cells as previously confirmed by Western blotting (12). MCF-10A cell lysates were collected from infected

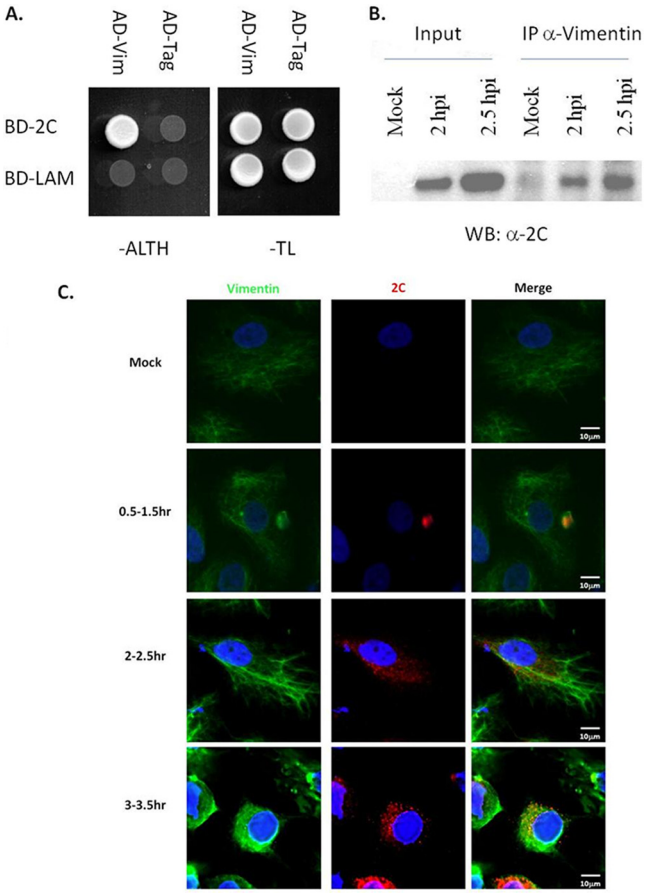

FIG 1 Protein-protein interaction of FMDV 2C with bovine vimentin in the yeast two-hybrid system (A), coimmunoprecipitation (B), and immunofluorescence staining (C). (A) Yeast strain AH109 was transformed with GAL4-binding domain (BD) fused to FMDV 2C (BD-2C) or a negative control, human lamin C (BD-LAM). These strains were then transformed with GAL4 activation domain (AD) fused to vimentin (AD-Vim) or T antigen (AD-Tag) as indicated above each lane. Spots of strains expressing the indicated constructs containing 2×10^6 yeast cells were spotted on selective media to screen for protein-protein interaction in the yeast two-hybrid system: either SD Ade/His/Leu/Trp plates (ALTH) or nonselective SD_Leu/Trp (_TL) for plasmid maintenance only. (B) Western blot probing for FMDV 2C (~40 kDa). Input cell lysate from mock-infected or FMDV-infected (at 2 and 2.5 hpi) preparations. Coimmunoprecipitation of FMDV 2C from mock-infected or FMDV O1C-infected (at 2 and 2.5 hpi) cell lysates performed using a MAb specific to vimentin. (C) Analysis of the distribution of vimentin and FMDV 2C proteins in MCF-10A cells. Cells were infected or mock infected with FMDV O1C and processed by immunofluorescence staining as described in Materials and Methods. FMDV 2C was detected with MAb 3D10 and visualized with Alexa Fluor 594 (red). Vimentin was detected with MAb V6630 and visualized with Alexa Fluor 488 (green). Yellow indicates colocalization of Alexa Flour 594 and 488 in the merged image.

or mock-infected cells and immunoprecipitated with an anti-vimentin MAb (V6630), followed by a Western blot specific for FMDV 2C protein (3D10). A single band was observed at the correct molecular mass (40 kDa) for 2C, indicating that 2C and vimentin interact during FMDV infection, confirming the results obtained by the yeast two-hybrid methodology (Fig. 1B). Several attempts were made to do the reverse coimmunoprecipitation methodology by pulling down FMDV protein 2C and blotting for vimentin; however, the vimentin band is masked by a background band belonging to the immunoglobulin heavy chain of the MAb used in the immunoprecipitation step, so the results were inconclusive.

Vimentin forms a cage-like structure around 2C. To further confirm that vimentin interacts with 2C during FMDV infection, we analyzed the location of both proteins at different times postinfection by using double-label immunofluorescence in cells that were infected with FMDV. MCF-10A cells were infected with an MOI of 10 or mock infected with FMDV O1C. Cells were then fixed on glass coverslips at 30-min intervals after infection up to 4 hpi and stained using MAbs that exhibit specific fluorescence for either FMDV 2C or vimentin (3D10 and V6630, respectively). The results indicated that there was a clear transitory colocalization of FMDV 2C and vimentin proteins between 0.5 and 1.5 hpi, with vimentin forming a cage-like structure around 2C in all cells infected with FMDV (Fig. 1C). Interestingly, similar cage-like structures formed by vimentin-associated virus proteins have been observed in cells infected with African swine fever virus, vaccinia virus, and some iridoviruses (25, 40, 41). Therefore, initial results obtained by yeast two-hybrid showing interaction between virus 2C and cellular vimentin were confirmed in FMDV-infected cells by two independent methodologies.

Vimentin is degraded in FMDV-infected cells. To determine whether FMDV infection by itself has an effect on the levels of vimentin expression, MCF-10A cells were infected with an MOI of 1 with FMDV O1C, and cell lysates were collected every 30 min during the course of infection up to 4 hpi. Samples were tested by Western blotting (Fig. 2A), and the expression levels of vimentin in the cell lysates were quantified using ImageJ software (obtained from the National Center for Biotechnology Information) using the recommended procedure to calculate relative density compared to a treated control (Fig. 2B). Expression of endogenous levels of actin was used to normalize vimentin values. The results demonstrated transitory increasing amounts (~20 to 40%) of vimentin in the infected cells between 1.5 to 3 hpi, with a decrease after 3 hpi. Accordingly, vimentin degradation products (50, 46, and 29 kDa) began to appear at 2.5 hpi, increasing for the remainder of the infection, possibly suggesting that this degradation could be how the vimentin cage is resolved after the initial formation around 2C.

Expression of dominant-negative forms of vimentin decrease viral yield. To assess the role of vimentin in FMDV replication we attempted to manipulate the levels of vimentin in infected cells. MCF-10A cells were transfected with either a plasmid encoding a full-length vimentin gene (HA-vimentin), used to increase cellular levels of vimentin, or a truncated form of the vimentin gene (HA-DN-vimentin, truncated at amino acid residue 134 compared to the full-length vimentin at 466 amino acids) that acts as a dominant-negative form of vimentin (42–44), to disrupt vimentin function. Both constructs had an HA tag for detection of the respective protein products by Western blotting. Cell lysates were taken 19 h after transfection and assessed by Western blotting for HA (Fig. 3B), demonstrating the expression of either construct (Fig. 3B). Furthermore, MCF-10A cells overexpressing HA-vimentin, HA-DN-vimentin or a control plasmid containing GFP were infected 19 h posttransfection infected with an MOI of 0.1 with FMDV O1C and virus yields in the extracellular medium were assessed at 0, 5, and 24 hpi. The results demonstrated that while overexpression of vimentin does not affect virus replication, cells overexpressing the dominant-negative form of vimentin presented a significant reduction (>2 logs) in virus titer compared to cells overexpressing full-length vimentin or an GFP-expressing control (Fig. 3A).

To determine whether the decrease in viral replication in the presence of a dominant-negative form of vimentin was related to the inability of the vimentin cage to form around 2C during infection, double-label immunofluorescence microscopy against 2C and vimentin in cells overexpressing truncated forms of vimentin and infected with FMDV was performed. HA-DN-vimentin-transfected MCF-10A cells were infected with an MOI of 10 or mock infected with FMDV O1C. Cells were then fixed on glass coverslips at the indicated time points and stained using MAbs that demonstrate specific fluorescence for either FMDV 2C or vimentin (Fig. 3C). The results indicated that the vimentin cage

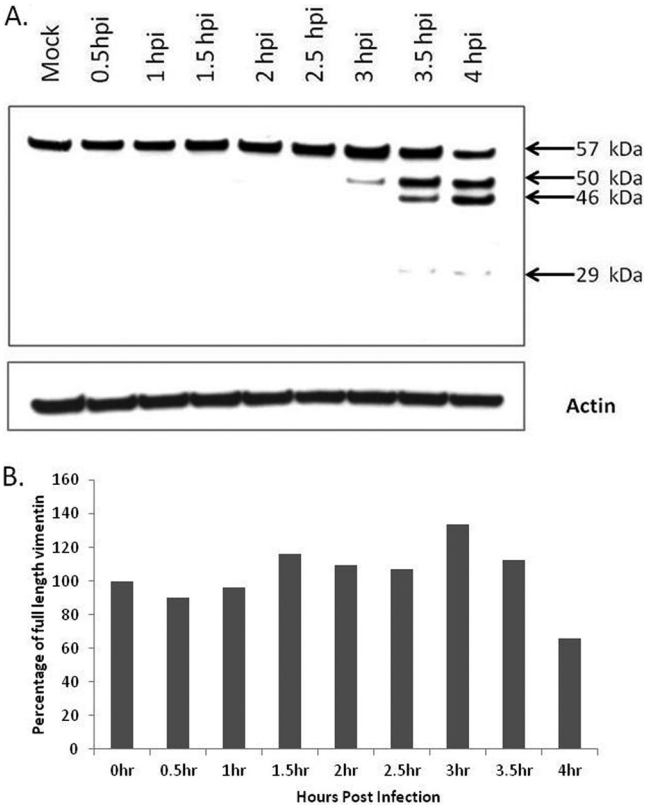

FIG 2 Analysis of vimentin expression in FMDV-infected MCF-10A cells. (A) Cells were infected with FMDV O1C and at different times postinfection, cell were lysed in RIPA buffer and assessed by Western blotting with a specific MAb V6630 vimentin. Full-length Vimentin is ~57 kDa, and the breakdown fragments are 50, 46, and 29 kDa, as indicated by the arrows. Actin (MAb1501) is shown as a loading control. (B) Quantification of vimentin bands in a representative Western blot shown in panel A using ImageJ analysis software using actin levels to normalize each sample.

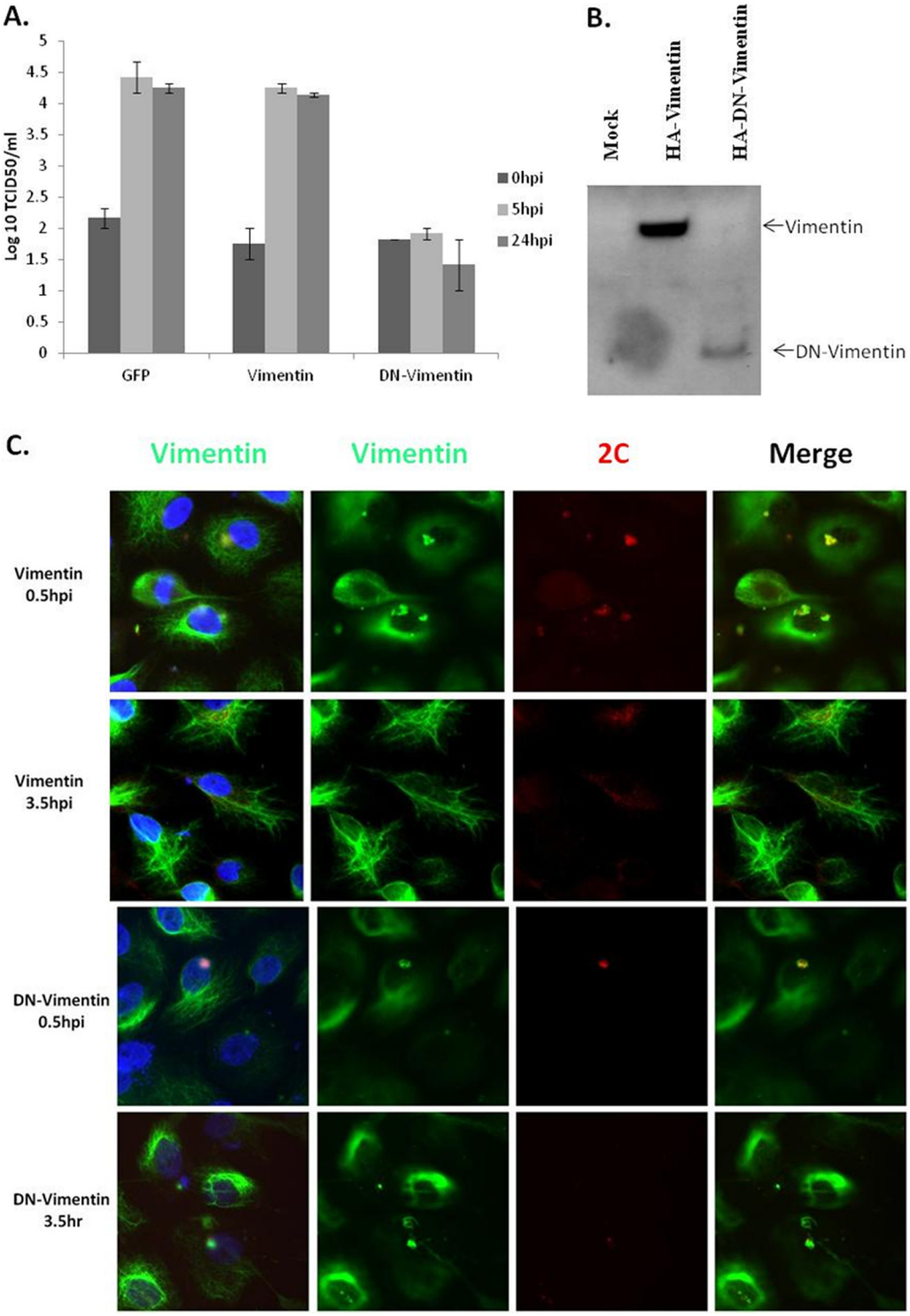

FIG 3 Effect of vimentin or truncated forms of vimentin on FMDV replication. MCF-10A cells were transfected with a plasmid encoding vimentin (pvimentin-HA), DN-vimentin (pDN-vimentin-HA), or GFP (pGFP) and then at 19 h posttransfection were infected with an MOI of 10 with FMDV O1C. (A) Virus yield is expressed as the $\log_{10}$ 50% tissue culture infective doses ($TCID_{50}$)/ml. Each value represents the mean and standard deviation from two independent experiments. The sensitivity of virus detection was $\geq\log_{10}$ 1.8 $TCID_{50}$/ml. (B) Expression levels of vimentin and DN-vimentin detected by Western blotting using an anti-HA MAb (DW2), with the correct predicted sizes of ~57 kDa for vimentin and 15 kDa for DN-vimentin. (C) Analysis of the distribution of vimentin and FMDV 2C proteins at different times postinfection. Cells were processed by immunofluorescence staining as described in Materials and Methods. FMDV 2C was detected with MAb 3D10 and visualized with Alexa Fluor 594 (red). Vimentin was detected with MAb V6630 and visualized with Alexa Fluor 488 (green). Yellow indicates colocalization of Alexa Flour 594 and 488 in the merged image.

was formed in the presence of a dominant-negative form of vimentin at 0.5 hpi as it happens in a normal viral infection. However, the vimentin cage, which is normally resolved by 2 hpi, remained unaltered in cells containing the DN form of vimentin at least until 4 hpi. At 4 hpi, an apparent decrease in the intensity of 2C fluorescence was observed in vimentin cages, with some vimentin cages having undetectable levels of 2C, suggesting that 2C could be degraded if the cage is not resolved. Therefore, expression of a DN form of vimentin appears to correlate with permanence of the vimentin cage and a significant reduction in viral yield.

An intact vimentin but not microtubule pathway is required for FMDV replication. Vimentin has a radial organization, where filaments typically extend outward from the cell center and it has been shown that vimentin structures can move on microtubules (45, 46). Since expression of truncated forms of vimentin has a significant effect on virus replication, it is interesting to evaluate whether the integrity of the endogenous vimentin and associated structures directly affect FMDV replication. To assess this issue, we carried out pharmacological experiments using nocodazole and acrylamide, which specifically disrupt the microtubules and the vimentin intermediate filament networks, respectively (47, 48). Mock-treated MCF-10A cells present an intact vimentin structure dispersed throughout the cytoplasm, while in the presence of nocodazole there was a redistribution of vimentin toward the nucleus (Fig. 4B). This observation concurs with a previous study showing that vimentin intermediate filaments are organized by the microtubule network (48). The addition of acrylamide causes the disruption of vimentin. This effect was observed in MCF-10A cells treated with acrylamide (Fig. 4B).

To test the effect of the changes of vimentin distribution on virus replication, MCF-10A cells were pretreated for 30 min with either nocodazole or acrylamide and then infected with an MOI of 0.1 of FMDV O1C (Fig. 4A). Interestingly, treatment with nocodazole had no significant effect on viral replication, agreeing with previous studies (49). However, treatment with 400 μM acrylamide resulted in a consistent 10-fold decrease in virus titers. Assessment of cell viability after acrylamide treatment demonstrated a viability of 94.5% ± 0.03%, by using an MTT assay. Thus, vimentin disruption resulted in decreased virus titers, supporting the hypothesis that an intact vimentin network is required for viral replication while disruption of the microfilament network has no effect on FMDV replication.

Alanine scanning of FMDV 2C reveals binding site for vimentin. To evaluate the importance of the binding between 2C and vimentin to FMDV replication, we tried to identify amino acid residues within 2C that directly mediate the interaction with vimentin. To determine the binding site(s) for vimentin present in 2C, an alanine scanning mutagenesis approach was used. Using site-directed mutagenesis, 46 mutant 2C proteins were constructed (35) in the yeast two-hybrid system to contain a stretch of seven amino acids that were changed from their native amino acid to alanine residues (Fig. 5A). Each of these mutated 2C proteins were assessed in their ability to bind vimentin in the yeast two-hybrid system. 2C proteins containing mutations in areas 12 and 13 were unable to bind vimentin (Fig. 5B). To ensure that all 2C alanine mutants were still able to be expressed in the yeast two-hybrid system, protein Beclin-AD (12) (another host protein that was detected as a binding partner for 2C) was used as an internal control. Beclin-AD was able to interact with all 2C mutants lacking vimentin binding, demonstrating that mutating these areas specifically interrupted the binding between vimentin and 2C.

Binding between FMDV 2C and host vimentin appears critical for virus replication. Reverse genetics was used to assess the effect of 2C mutations identified as critical in mediating the interaction between 2C and vimentin. Infectious clones (ICs) of FMDV O1C (31) containing areas of 2C harboring the same alanine substitutions shown to alter 2C-vimentin reactivity in the yeast two-hybrid were constructed. Those IC constructs were then used to produce the corresponding RNAs by *in vitro* transcription, which were then used in cell transfections to produce their respective FMDV progeny. Although transfection with parental FMDV O1C RNA produced viable virus progeny, mutants 2C-12 and 13 (Fig. 5A) consistently produced nonviable virus. It is possible that these mutations, besides altering the reactivity with vimentin, could be detrimental to the virus for other as yet unidentified reasons. Therefore, a more detailed mapping of the area of interaction between vimentin and 2C was performed with the aim of reducing the number of amino acids mutated and still disrupt the interaction. The regions in 2C-12 and 13 were further subdivided into three separate subareas (comprising two to three residues each), which were individually assessed in their reactivity with vimentin in the yeast two-hybrid system (Fig. 5C). However, none of the smaller mutated regions resulted in the lost ability to bind vimentin, suggesting that multiple contact points within the seven residue stretch is needed for disruption of 2C binding to vimentin.

DISCUSSION

The complex cellular pathways and mechanisms that FMDV manipulates to facilitate viral replication and to evade host defenses are not completely understood. One potential mechanism FMDV may use to manipulate the host cell involves interaction with cellular proteins to modify their function, thus changing the natural cellular pathway. For example, we previously demonstrated that FMDV 2C could manipulate the autophagy pathway by binding to Beclin1, resulting in the inability of autophagosomes to fuse with lysosomes, favoring virus survival. We report here that FMDV nonstructural protein 2C binds to host class III intermediate filament vimentin. A yeast two-hybrid model was used to identify vimentin as a specific protein binding partner for viral 2C. We also demonstrated coimmunoprecipitation of 2C with vimentin from FMDV-infected cell extracts as well as colocalization of 2C with vimentin in cells infected with FMDV.

The importance of vimentin during viral infection has been described in a wide range of viruses. For example, in cowpea mosaic virus and Japanese encephalitis virus (JEV), surface-expressed vimentin is necessary for efficient viral entry (18, 50, 51). An increase of vimentin expression is observed during infection with Hepatitis C virus (HCV) (52), T-cell leukemia virus type I (24) and rabies virus (RV) (53). Alternatively, during infection with retroviruses, including human immunodeficiency viruses and bovine leukemia virus (BLV), the viral-encoded protease specifically cleaves vimentin; however, the function of vimentin cleavage is still unknown (22, 54, 55). It is possible that vimentin is cleaved to change the global cell structure or to prevent intracellular cell signaling as seen with Epstein-Barr virus protein LMP1 which causes the disruption of vimentin to modulate cell signaling (56). Studies of other viruses have shown that viral proteins directly bind vimentin as observed with dengue virus nonstructural protein 1, where vimentin binding is critical for virus replication (57),

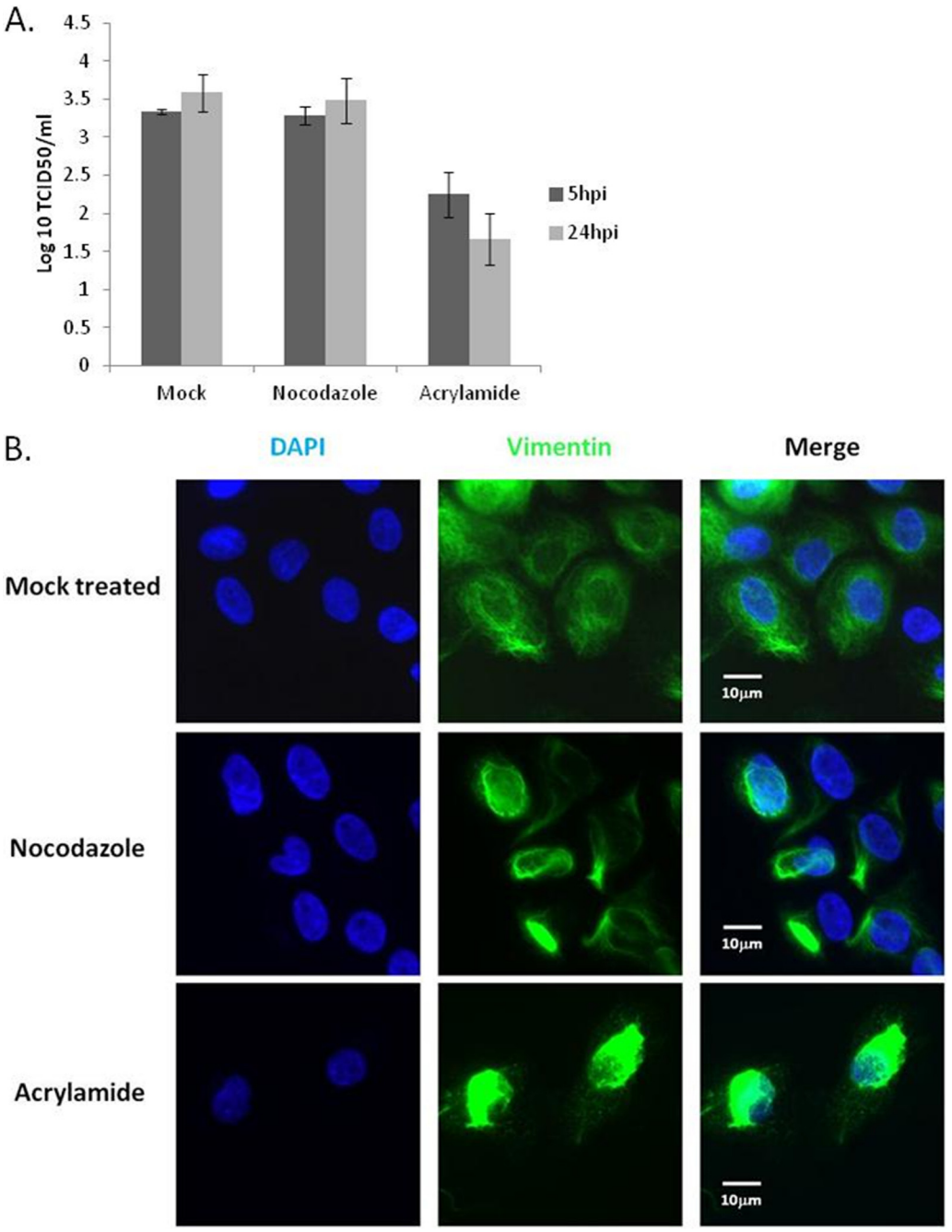

FIG 4 Effect of nocodazole and acrylamide treatment on MCF-10A cell cultures infected with FMDV. MCF-10A cell cultures were treated with either drug for 1 h and then infected with FMDV 01C infected with an MOI of 0.1. (A) Virus yield was assessed at the indicated times postinfection and expressed as $\log_{10}$ $TCID_{50}$/ml. Each value represents the mean and standard error from two independent experiments. The sensitivity of virus detection was $\geq \log_{10}$ 1.8 $TCID_{50}$/ml. (B) Analysis of the distribution of vimentin in MCF-10A cells treated with either nocodazole (5 μm) or acrylamide (400 μm). Cells were treated and then processed by immunofluorescence staining as described in Materials and Methods using a vimentin-specific MAb (V6630) and visualized with Alexa Fluor 488 (green).

and in bluetongue virus protein VP2, where binding of vimentin is necessary for viral egress (19). In African swine fever virus, it has been shown that vimentin is arranged around viral factories, forming a cage-like structure, which may aid to isolate viral proteins from the rest of the cell (25).

In previous studies by Armer et al. (5), some degree of vimentin rearrangement was observed with FMDV, but their studies were limited to time points late during infection, where they observed a lack of vimentin in areas of the cell cytoplasm where virus was being produced. We observed that early during infection, starting at 0.5 hpi, cellular vimentin colocalized and formed a cage-like structure around FMDV protein 2C, and that later during infection this cage-like structure disappeared as viral infection progressed. We also observed that as infection progressed, vimen-

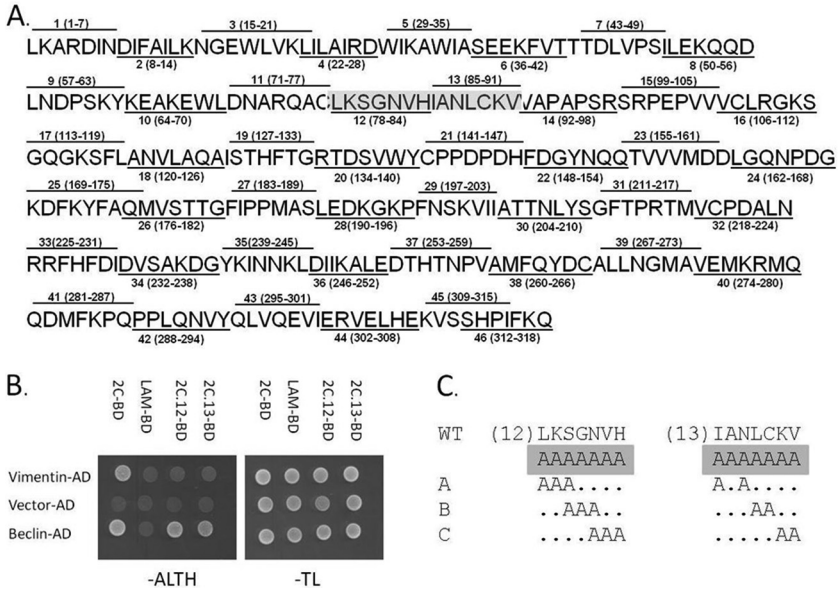

FIG 5 Scheme showing FMDV 2C alanine mutants used in the present study. (A) Each alanine 2C mutant name is followed, in parentheses, by the amino acid residues mutated for that mutant. All indicated residues were mutated to an alanine. The highlighted 2C mutants represent mutations that resulted in lack of binding of 2C to vimentin in the yeast two-hybrid system. (B) Yeast strain AH109 was transformed with either GAL4-binding domain (BD) fused to FMDV 2C (2C-BD), the indicated FMDV 2C mutation, or as a negative control human lamin C (LAM BD). These strains were then transformed with GAL4 activation domain (AD) fused to vimentin (Vimentin-AD) or Beclin1 (Beclin1-AD) as indicated above. Strains expressing the indicated constructs containing 2×10^6 yeast cells were spotted onto selective media to evaluate protein-protein interaction in the yeast two-hybrid system, using either SD−Ade/His/Leu/Trp plates (−ALTH) or nonselective SD−Leu/Trp plates (−TL) for plasmid maintenance only. (C) Subdivisions (labeled A, B, and C) of the original alanine scanning mutants 12 and 13.

tin degradation occurred, possibly explaining the previously observed vimentin rearrangement at late time points during infection (33).

To determine whether an intact vimentin pathway is required for FMDV infection, we utilized overexpression plasmids containing either the wild-type or a truncated form of vimentin and showed that in the presence of truncated vimentin the virus exhibited reduced virus replication. Along with this observation, cells expressing the truncated vimentin form still contained vimentin cages but, in contrast to what is observed in cells containing wild-type vimentin, cages were never able to be resolved. It may be that the formation of the vimentin cage is part of an initial cellular response to isolate the virus from the rest of the cell, consequently serving as an initial site of replication. If so, then it is possible that once enough viral replication or viral protein 2C is produced the vimentin cage may be resolved. However, in the presence of truncated vimentin one possibility is that 2C gets bound to the truncated vimentin, and this could affect the ability for the vimentin cage to be resolved, which could allow the virus proteins in the vimentin cage to eventually be degraded by the host cell. However, it is still unclear how the vimentin cage is able to form in the presence of a dominant-negative form of vimentin; it is possible that the formation of the cage does not rely on full-length vimentin or the normal vimentin pathway and that an intact vimentin pathway is only required to resolve the vimentin cage.

Chemical disruption of vimentin using acrylamide resulted in decreased viral yield, supporting the hypothesis that an intact vimentin network is required for viral replication. Conversely, in agreement with previous reports (49), chemical disruption of microfilaments using nocodazole does not affect viral replication, which is surprising since disruption of the microfilament network also disrupts the vimentin filament network, as noted in Fig. 4B. However, this fact is not unique to FMDV, since a similar observation has been seen with dengue virus (21), where the virus requires an intact vimentin network, but not a microtubule network, for efficient viral replication.

Although it appears that the 2C-vimentin interaction is necessary for virus replication and that an intact vimentin pathway is required for a successful FMDV infection, the precise mechanism governing vimentin cage formation and its later resolution, allowing FMDV to progress with replication, remains to be elucidated. During ASFV infection, it has been shown that vimentin recruits the virus to the microtubule-organizing center (MTOC); however, the specific reason for this is unknown. As a marker for MTOC we tested if the vimentin cage colocalizes with γ-tubulin; no clear colocalization with the vimentin cage formed by FMDV and γ-tubulin could be observed (data not shown), suggesting that the formation of the vimentin cage during FMDV infection may play a different role than that of the vimentin cage during ASFV infection. This is in agreement with previous studies from Armer et al. (5) that show the MTOC remains intact during FMDV infection while γ-tubulin is lost from the MTOC later in infection, suggesting that the MTOC is not involved in FMDV replication. Although the precise role played by the formation of the vimentin cage is unknown, it is possible that its initial formation is to protect the cell from the accumulation of 2C or, conversely, to prevent 2C from being degraded by the cellular defense machinery. Alternatively, it could also be possible that the vimentin cage may provide a physical scaffold that is required for the

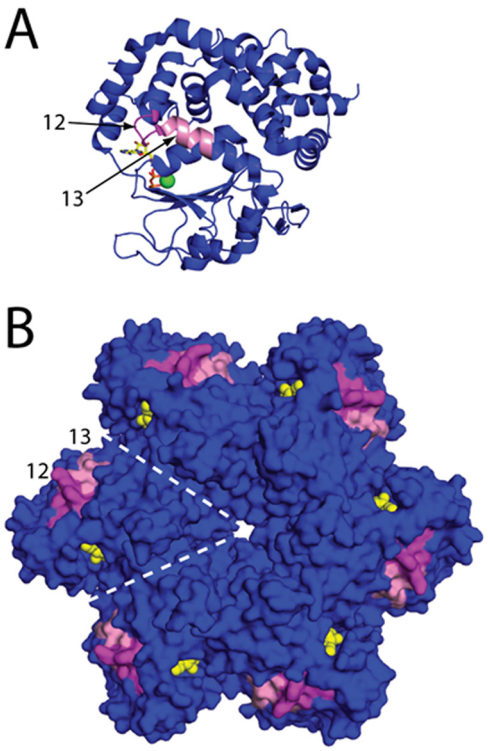

FIG 6 Mapping the alanine scanning blocks 12 (magenta, labeled) and 13 (pink, labeled) effecting vimentin binding with FMDV 2C to corresponding regions on the crystal structure of the AAA+ homolog SV40 LT (PDB ID 1SVM) to assess potential accessibility and localize the blocks in the context of the AAA+ fold. The bound ATP is shown in a yellow/orange and the associated magnesium ion in green. (A) Richardson diagram of an SV40 LT monomer with bound ATP in stick representation. (B) Accessible surface diagram of the SV40 LT hexamer with bound ATP as a van der Waals surface. Dashed white lines give the approximate bounds of one monomer within the hexamer.

initial virus replication, preventing viral proteins from diffusing out into the cytoplasm. Further work is necessary to better characterize this phenomenon.

To gain some potential insight into how the alanine mutations in 2C could affect the binding to vimentin, we performed bioinformatic analysis to map to corresponding regions of homologous proteins with known crystal structures to assess potential accessibility and their location in the context of the AAA+ fold. The regions in 2C responsible for vimentin binding map to a region that could be potentially exposed in the previously determined hexameric state of 2C (58), suggesting that these two areas may be directly accessible for vimentin binding in 2C (Fig. 6). We also note that the region of SV40 LT corresponding to alanine scanning block 12 makes direct contact with ATP in the SV40 LT crystal structure (59) (Fig. 6). However, in order to determine exactly how vimentin interacts structurally and functionally with these potential areas of 2C, further experiments, and perhaps the crystal structure of FMDV 2C would have to be performed.

The results reported here identify, for the first time, cellular host protein vimentin as an interaction partner for FMDV viral protein 2C. This interaction appears to be critical for virus growth since FMDV genomes harboring 2C mutations that disrupted the interaction between 2C and vimentin resulted in viruses unable to replicate in cell cultures. Importantly, the 2C-vimentin interaction appears to somehow modulate the host cell environment to allow for viral replication. This presents new possibilities of exploration for FMDV pathogenesis, and perhaps new insights into how the virus is able to form and later resolve the vimentin cage, providing novel starting points toward designing novel therapeutic strategies that target the cellular vimentin pathway. In addition, further work still needs to be done to understand other host protein-viral protein relationships and how the virus utilizes or avoids specific cellular pathways for its own survival.

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REFERENCES

1. **Carrillo C, Tulman ER, Delhon G, Lu Z, Carreno A, Vagnozzi A, Kutish GF, Rock DL.** 2005. Comparative genomics of foot-and-mouth disease virus. J. Virol. **79**:6487–6504.
2. **Kitching RP, Knowles NJ, Samuel AR, Donaldson AI.** 1989. Development of foot-and-mouth disease virus strain characterization: a review. Trop. Anim. Health Prod. **21**:153–166.
3. **Grubman MJ, Baxt B.** 2004. Foot-and-mouth disease. Clin. Microbiol. Rev. **17**:465–493.
4. **Grubman MJ, Baxt B.** 1982. Translation of foot-and-mouth disease virion RNA and processing of the primary cleavage products in a rabbit reticulocyte lysate. Virology **116**:19–30.
5. **Armer H, Moffat K, Wileman T, Belsham GJ, Jackson T, Duprex WP, Ryan M, Monaghan P.** 2008. Foot-and-mouth disease virus, but not bovine enterovirus, targets the host cell cytoskeleton via the nonstructural protein 3Cpro. J. Virol. **82**:10556–10566.
6. **Bienz K, Egger D, Pasamontes L.** 1987. Association of polioviral proteins of the P2 genomic region with the viral replication complex and virus-induced membrane synthesis as visualized by electron microscopic immunocytochemistry and autoradiography. Virology **160**:220–226.
7. **Bienz K, Egger D, Wolff DA.** 1973. Virus replication, cytopathology, and lysosomal enzyme response of mitotic and interphase Hep-2 cells infected with poliovirus. J. Virol. **11**:565–574.
8. **Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D, Bienz K.** 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virol. **76**:5974–5984.
9. **Monaghan P, Cook H, Jackson T, Ryan M, Wileman T.** 2004. The ultrastructure of the developing replication site in foot-and-mouth disease virus-infected BHK-38 cells. J. Gen. Virol. **85**:933–946.
10. **Pedersen KW, van der Meer Y, Roos N, Snijder EJ.** 1999. Open reading frame 1a-encoded subunits of the arterivirus replicase induce endoplasmic reticulum-derived double-membrane vesicles which carry the viral replication complex. J. Virol. **73**:2016–2026.
11. **Schwartz M, Chen J, Lee WM, Janda M, Ahlquist P.** 2004. Alternate, virus-induced membrane rearrangements support positive-strand RNA virus genome replication. Proc. Natl. Acad. Sci. U. S. A. **101**:11263–11268.
12. **Gladue DP, O'Donnell V, Baker-Branstetter R, Holinka LG, Pacheco JM, Fernandez-Sainz I, Lu Z, Brocchi E, Baxt B, Piccone ME, Rodriguez L, Borca MV.** 2012. Foot-and-mouth disease virus nonstructural protein 2C interacts with beclin1, modulating virus replication. J. Virol. **86**:12080–12090.
13. **Moffat K, Knox C, Howell G, Clark SJ, Yang H, Belsham GJ, Ryan M, Wileman T.** 2007. Inhibition of the secretory pathway by foot-and-mouth disease virus 2BC protein is reproduced by coexpression of 2B with 2C, and the site of inhibition is determined by the subcellular location of 2C. J. Virol. **81**:1129–1139.
14. **O'Donnell V, Pacheco JM, LaRocco M, Burrage T, Jackson W, Rodriguez LL, Borca MV, Baxt B.** 2012. Foot-and-mouth disease virus utilizes an autophagic pathway during viral replication. Virology **410**:142–150.

15. **Earl RT, Mangiapane EH, Billett EE, Mayer RJ.** 1987. A putative protein-sequestration site involving intermediate filaments for protein degradation by autophagy: studies with transplanted Sendai-viral envelope proteins in HTC cells. Biochem. J. **241**:809–815.
16. **Mayer RJ, Gaskell MJ, Earl R, Billett EE, Mangiapane HE, Fernig D, Doherty FJ.** 1986. Intracellular protein catabolism: evidence for sequestration of proteins into an intermediate-filament fraction before lysosomal degradation. Biomed. Biochim. Acta **45**:1591–1602.
17. **Koudelka KJ, Destito G, Plummer EM, Trauger SA, Siuzdak G, Manchester M.** 2009. Endothelial targeting of cowpea mosaic virus (CPMV) via surface vimentin. PLoS Pathog. **5**:e1000417. doi:10.1371/journal.ppat.1000417.
18. **Das S, Ravi V, Desai A.** 2011. Japanese encephalitis virus interacts with vimentin to facilitate its entry into porcine kidney cell line. Virus Res. **160**:404–408.
19. **Bhattacharya B, Noad RJ, Roy P.** 2007. Interaction between Bluetongue virus outer capsid protein VP2 and vimentin is necessary for virus egress. Virol. J. **4**:7.
20. **Risco C, Rodriguez JR, Lopez-Iglesias C, Carrascosa JL, Esteban M, Rodriguez D.** 2002. Endoplasmic reticulum-Golgi intermediate compartment membranes and vimentin filaments participate in vaccinia virus assembly. J. Virol. **76**:1839–1855.
21. **Chen W, Gao N, Wang JL, Tian YP, Chen ZT, An J.** 2008. Vimentin is required for dengue virus serotype 2 infection but microtubules are not necessary for this process. Arch. Virol. **153**:1777–1781.
22. **Shoeman RL, Mothes E, Honer B, Kesselmeier C, Traub P.** 1991. Effect of human immunodeficiency virus type 1 protease on the intermediate filament subunit protein vimentin: cleavage, in vitro assembly and altered distribution of filaments in vivo following microinjection of protease. Acta Histochem. Suppl. **41**:129–141.
23. **Belin MT, Boulanger P.** 1987. Processing of vimentin occurs during the early stages of adenovirus infection. J. Virol. **61**:2559–2566.
24. **Lilienbaum A, Duc Dodon M, Alexandre C, Gazzolo L, Paulin D.** 1990. Effect of human T-cell leukemia virus type I tax protein on activation of the human vimentin gene. J. Virol. **64**:256–263.
25. **Stefanovic S, Windsor M, Nagata KI, Inagaki M, Wileman T.** 2005. Vimentin rearrangement during African swine fever virus infection involves retrograde transport along microtubules and phosphorylation of vimentin by calcium calmodulin kinase II. J. Virol. **79**:11766–11775.
26. **Murti KG, Chen M, Goorha R.** 1985. Interaction of frog virus 3 with the cytomatrix III. Role of microfilaments in virus release. Virology **142**:317–325.
27. **Johnston JA, Ward CL, Kopito RR.** 1998. Aggresomes: a cellular response to misfolded proteins. J. Cell Biol. **143**:1883–1898.
28. **Chien CT, Bartel PL, Sternglanz R, Fields S.** 1991. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. U. S. A. **88**:9578–9582.
29. **Fields S, Song O.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340**:245–246.
30. **Schneider CA, Rasband WS, Eliceiri KW.** NIH Image to ImageJ: 25 years of image analysis. Nat. Methods **9**:671–675.
31. **Borca MV, Pacheco JM, Holinka LG, Carrillo C, Hartwig E, Garriga D, Kramer E, Rodriguez L, Piccone ME.** 2011. Role of arginine-56 within the structural protein VP3 of foot-and-mouth disease virus (FMDV) O1 Campos in virus virulence. Virology **422**:37–45.
32. **Mason PW, Rieder E, Baxt B.** 1994. RGD sequence of foot-and-mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody-dependent enhancement pathway. Proc. Natl. Acad. Sci. U. S. A. **91**:1932–1936.
33. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403–410.
34. **Soding J.** 2005. Protein homology detection by HMM-HMM comparison. Bioinformatics **21**:951–960.
35. **Fields S, Sternglanz R.** 1994. The two-hybrid system: an assay for protein-protein interactions. Trends Genet. **10**:286–292.
36. **Gao Y, Sztul E.** 2001. A novel interaction of the Golgi complex with the vimentin intermediate filament cytoskeleton. J. Cell Biol. **152**:877–894.
37. **Styers ML, Kowalczyk AP, Faundez V.** 2005. Intermediate filaments and vesicular membrane traffic: the odd couple's first dance? Traffic **6**:359–365.
38. **Styers ML, Salazar G, Love R, Peden AA, Kowalczyk AP, Faundez V.** 2004. The endo-lysosomal sorting machinery interacts with the intermediate filament cytoskeleton. Mol. Biol. Cell **15**:5369–5382.
39. **Doherty FJ, Wassell JA, Mayer RJ.** 1987. A putative protein-sequestration site involving intermediate filaments for protein degradation by autophagy. Studies with microinjected purified glycolytic enzymes in 3T3-L1 cells. Biochem. J. **241**:793–800.
40. **Ferreira LR, Moussatche N, Moura Neto V.** 1994. Rearrangement of intermediate filament network of BHK-21 cells infected with vaccinia virus. Arch. Virol. **138**:273–285.
41. **Heath CM, Windsor M, Wileman T.** 2001. Aggresomes resemble sites specialized for virus assembly. J. Cell Biol. **153**:449–455.
42. **Goldman RD, Khuon S, Chou YH, Opal P, Steinert PM.** 1996. The function of intermediate filaments in cell shape and cytoskeletal integrity. J. Cell Biol. **134**:971–983.
43. **Kural C, Serpinskaya AS, Chou YH, Goldman RD, Gelfand VI, Selvin PR.** 2007. Tracking melanosomes inside a cell to study molecular motors and their interaction. Proc. Natl. Acad. Sci. U. S. A. **104**:5378–5382.
44. **Whipple RA, Balzer EM, Cho EH, Matrone MA, Yoon JR, Martin SS.** 2008. Vimentin filaments support extension of tubulin-based microtentacles in detached breast tumor cells. Cancer Res. **68**:5678–5688.
45. **Gyoeva FK, Gelfand VI.** 1991. Coalignment of vimentin intermediate filaments with microtubules depends on kinesin. Nature **353**:445–448.
46. **Liao G, Gundersen GG.** 1998. Kinesin is a candidate for cross-bridging microtubules and intermediate filaments. Selective binding of kinesin to detyrosinated tubulin and vimentin. J. Biol. Chem. **273**:9797–9803.
47. **Arocena M.** 2006. Effect of acrylamide on the cytoskeleton and apoptosis of bovine lens epithelial cells. Cell Biol. Int. **30**:1007–1012.
48. **Vasquez RJ, Howell B, Yvon AM, Wadsworth P, Cassimeris L.** 1997. Nanomolar concentrations of nocodazole alter microtubule dynamic instability *in vivo* and *in vitro*. Mol. Biol. Cell **8**:973–985.
49. **O'Donnell V, Larocco M, Baxt B.** 2008. Heparan sulfate-binding foot-and-mouth disease virus enters cells via caveola-mediated endocytosis. J. Virol. **82**:9075–9085.
50. **Liang JJ, Yu CY, Liao CL, Lin YL.** 2011. Vimentin binding is critical for infection by the virulent strain of Japanese encephalitis virus. Cell Microbiol. **13**:1358–1370.
51. **Plummer EM, Thomas D, Destito G, Shriver LP, Manchester M.** Interaction of cowpea mosaic virus nanoparticles with surface vimentin and inflammatory cells in atherosclerotic lesions. Nanomedicine (Lond.) **7**:877–888.
52. **Ghosh S, Ahrens WA, Phatak SU, Hwang S, Schrum LW, Bonkovsky HL.** Association of filamin A and vimentin with hepatitis C virus proteins in infected human hepatocytes. J. Viral Hepat. **18**:e568–e577.
53. **Wang X, Zhang S, Sun C, Yuan ZG, Wu X, Wang D, Ding Z, Hu R.** Proteomic profiles of mouse neuro N2a cells infected with variant virulence of rabies viruses. J. Microbiol. Biotechnol. **21**:366–373.
54. **Shoeman RL, Honer B, Stoller TJ, Kesselmeier C, Miedel MC, Traub P, Graves MC.** 1990. Human immunodeficiency virus type 1 protease cleaves the intermediate filament proteins vimentin, desmin, and glial fibrillary acidic protein. Proc. Natl. Acad. Sci. U. S. A. **87**:6336–6340.
55. **Snasel J, Shoeman R, Horejsi M, Hruskova-Heidingsfeldova O, Sedlacek J, Ruml T, Pichova I.** 2000. Cleavage of vimentin by different retroviral proteases. Arch. Biochem. Biophys. **377**:241–245.
56. **Meckes DG, Jr, Menaker NF, Raab-Traub N.** EBV LMP1 modulates lipid raft microdomains and the vimentin cytoskeleton for signal transduction and transformation. J. Virol., in press.
57. **Kanlaya R, Pattanakitsakul SN, Sinchaikul S, Chen ST, Thongboonkerd V.** 2010. Vimentin interacts with heterogeneous nuclear ribonucleoproteins and dengue nonstructural protein 1 and is important for viral replication and release. Mol. Biosyst. **6**:795–806.
58. **Sweeney TR, Cisnetto V, Bose D, Bailey M, Wilson JR, Zhang X, Belsham GJ, Curry S.** 2010. Foot-and-mouth disease virus 2C is a hexameric AAA+ protein with a coordinated ATP hydrolysis mechanism. J. Biol. Chem. **285**:24347–24359.
59. **Gai D, Zhao R, Li D, Finkielstein CV, Chen XS.** 2004. Mechanisms of conformational change for a replicative hexameric helicase of SV40 large tumor antigen. Cell **119**:47–60.