The human respiratory syncytial virus (HRSV) fusion (F) protein cytoplasmic tail (CT) and matrix (M) protein are key mediators of viral assembly, but the underlying mechanisms are poorly understood. A complementation assay was developed to systematically examine the role of the F protein CT in infectious virus production. The ability of F mutants with alanine substitutions in the CT to complement an F-null virus in generating infectious progeny was quantitated by flow cytometry. Two CT regions with impact on infectious progeny production were identified: residues 557 to 566 (CT-R1) and 569 to 572 (CT-R2). Substitutions in CT-R1 decreased infectivity by 40 to 85% and increased the level of F-induced cell-cell fusion but had little impact on assembly of viral surface filaments, which were believed to be virions. Substitutions in CT-R2, as well as deletion of the entire CT, abrogated infectious progeny production and impaired viral filament formation. However, CT-R2 mutations did not block but rather delayed the formation of viral filaments, which continued to form at a low rate and contained the viral M protein and nucleoprotein (N). Microscopy analysis revealed that substitutions in CT-R2 but not CT-R1 led to accumulation of M and F proteins within and at the perimeter of viral inclusion bodies (IBs), respectively. The accumulation of M and F at IBs and coincident strong decrease in filament formation and infectivity upon CT-R2 mutations suggest that F interaction with IBs is an important step in the virion assembly process and that CT residues 569 to 572 act to facilitate release of M-ribonucleoprotein complexes from IBs.
which show IBs and viral filaments in close proximity near the plasma membrane as well as an array of viral and cellular proteins that are shared between both structures (13, 18, 30, 31), suggest that IBs may constitute a type of scaffold from which viral filaments form. However, the structure and function of IBs are poorly understood. The presumed role of IBs in viral RNA transcription and replication is primarily based on the presence of viral RNA and the viral polymerase-associated proteins N, P, M2-1, and L (28, 29, 32, 33). Recently it was reported that IBs may also function to antagonize the innate immune response through N-mediated recruitment of cellular mediators (34), suggesting that these structures may serve multiple purposes. At a late stage in the infection cycle, the M protein is believed to target to IBs to shut down viral transcription and replication (1, 35, 36). However, the mechanics and timing of the steps that occur between the proposed shutdown of viral replication by the M protein and virion assembly are poorly understood. By analogy to the assembly process of other Mononegavirales, it is believed that the HRSV M protein interacts with the ribonucleoprotein (RNP) and with the viral glycoproteins to bring together the virion components. An interaction between M and G has been reported (37), but this interaction is not required for assembly as the virus replicates and forms viral filaments effectively in the absence of G (11, 38, 39). No reports have documented an interaction between M and the F protein CT, indicating that either HRSV may differ from other paramyxoviruses in this regard or the M-F interaction is short-lived or unstable.

In previous work, we generated an infectious virus lacking the F protein CT and documented the critical role of the CT in HRSV replication (3). To further dissect the functions of the CT domain in the context of a complete virus, we developed a flow cytometry assay based upon an F-null virus. Using this assay and IF microscopy, the impact of F protein CT mutations on infectious progeny production and the mechanisms underlying this impact were examined. The results suggest that the CT has multiple functions, one of which involves promoting the transition of assembly complexes from IBs to viral surface filaments.

MATERIALS AND METHODS

Cells and primary antibodies. HEp-2 and Vero cells were acquired from the American Type Culture Collection and grown in standard minimal essential medium (MEM) and Dulbecco’s modified Eagle’s medium (DMEM)-based growth medium containing 5% fetal bovine serum (FBS). Monoclonal antibodies L9 (anti-G) and A5 (anti-F) were provided by Edward Walsh (University of Rochester School of Medicine, Rochester, NY). Synagis (anti-F) and anti-N antibodies were acquired from MedImmune, Inc., and AbD Serotec, respectively. The anti-M antibody was a previously reported rabbit polyclonal peptide serum (2).

Construction and cloning of a codon-optimized F ORF and F protein CT modifications. The F open reading frame (ORF) of the HRSV A2 strain was codon optimized according to Haas et al. (40). Overlapping oligonucleotides (50 nucleotides [nt] with a 20-nt overlap), representing the entire codon-optimized ORF, were assembled and PCR amplified with flanking BsrGI and XhoI restriction sites. The product was cloned using BsrGI and XhoI restriction sites into plasmid pc-BX (a pcDNA3-derived plasmid modified to lack the T7 promoter and neomycin gene cassette and to contain an additional BsrGI restriction site). The codon-optimized F ORF was sequence verified. Errors due to the use of long primers were corrected using site-directed mutagenesis, and the final plasmid was named pc-FCT. A plasmid encoding an F protein lacking the CT (nucleotides encoding residues S554 to N574) was generated by PCR amplification of the codon-optimized F ORF using a reverse primer that anneals upstream of the CT, cloned into pc-BX, and named pc-F▵CT.

Alanine substitution mutants of pc-F▵CT were constructed by site-directed mutagenesis (Agilent), and sequence was verified.

Construction of an F-null cDNA. We previously reported a virus named RSV Fnull in which the F ORF was replaced with that of enhanced green fluorescent protein (GFP) (38). RSV Fnull contained artificial restriction sites to facilitate glycoprotein gene exchange. For this project, we constructed a second generation cDNA that lacks F expression and any artificial nucleotide sequences. First, using standard molecular biology techniques, a plasmid was constructed that contained a cDNA of the complete HRSV A2 strain (pRSV-A2). pRSV-A2 contained the same elements necessary for T7 polymerase-driven expression as in previous plasmids (11). pRSV-A2 was modified by replacing the F ORF with a spacer containing remote-cutting BsmBI sites (pRSV-ΔF/Bsm). Next, the ORFs encoding GFP and blue fluorescent protein (BFP) were PCR amplified using primers with flanking BsmBI sites, and products were digested with BsmBI and ligated into BsmBI-digested pRSV-ΔF/Bsm such that no artificial sequences remained other than the intended exact replacement of the F ORF with that of GFP or BFP. The resulting cDNAs were sequence verified and named pRSV-FnullGFP and pRSV-FnullBFP.

Recovery of infectious virus from cDNA. Infectious viruses were recovered from cDNAs pRSV-A2, pRSV-FnullGFP, and pRSV-FnullBFP as previously described (2, 38). The resulting wild-type (wt) virus, termed RSV-WT, was amplified in Vero cells. The resulting F-null viruses, termed RSV-FnullGFP and RSV-FnullBFP, were amplified in Vbac cells, which contain a baculovirus-derived functional F substitution (3). To verify the construct sequences, viral RNA was harvested from cells infected with the recovered viruses (passage 3 for RSV-WT and passage 4 for F-null viruses) and amplified by reverse transcription-PCR. Modified areas (GFP and BFP ORFs and surrounding intergenic regions) plus the M and G genes were verified by bulk nucleotide sequence analysis. For RSV-WT, the F gene sequence was also verified. Passage 3 stocks of RSV-WT virus and passage 4 and 5 stocks of the RSV-Fnull viruses were used for the described experiments.

Viral protein expression by cell ELISA. Relative protein expression levels were determined by cell enzyme-linked immunosorbent assay (ELISA) as previously described (2), with minor modifications. Briefly, transfected cells were fixed at 24 h posttransfection and permeabilized with 0.2% Triton X-100 (for N, G, and F) or 0.1% SDS (for M). Fixed, permeabilized cells were blocked and labeled with anti-N, -M, -G, or -F (A5) antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Fierce). After washing, cells were incubated in O-phenylenediamine-based substrate solution. At short time intervals, substrate aliquots were collected and added to 3 M sulfuric acid in 96-well plates to stop the reaction. The optical density at 490 nm (OD490) was determined in an ELISA plate reader.

Infectious progeny production assay. HEp-2 cells plated in six-well plates were transfected with plasmids encoding wt or mutant F proteins. At the end of a 5-h transfection, cells were infected with F-null virus at 1 PFU/cell for 2 h. At 34 hours postinfection (hpi), cells were scraped into the medium to capture cell-associated and released progeny virus and virus suspensions were stored at −80°C. At the time of assay, samples were thawed and mixed by extensive pipetting, and debris was removed by centrifugation at 800 × g. Supernatants were incubated on freshly plated HEp-2 cells in six-well plates (receiver cells) for 6 h and incubated at 37°C. At 24 hpi, receiver cells were trypsinized and fixed with 4% paraformaldehyde, and the percentage of GFP-expressing cells was determined by flow cytometry (50,000 cells per sample). In parallel wells transfected identically, the relative levels of total F expression were determined by cell ELISA as previously described (3), to ensure that F expression levels were similar.

Cell-cell fusion assay. To quantitate F-induced membrane fusion, a previously developed dual split-protein (DSP) assay was used (41). In the DSP assay, two plasmids (named DSP1 and DSP2), each expressing a portion of Renilla luciferase linked to a portion of GFP, are separately transfected and form functional luciferase-GFP only after transfected cells
are mixed and undergo membrane fusion (41). For each F mutant, subconfluent 293T cells (Eagle’s minimal essential medium [EMEM]-based medium) in 12-well plates were separately transfected with 1 μg DSP p1,7 plus 1 μg of F plasmid or with 1 μg DSP p1,7 alone. After transfection, cells transfected with F plasmids were grown in medium containing 250 nM RSV fusion inhibitor BMS-433771 (42) (a gift from Alios Biopharma). Twenty-four hours posttransfection, cells transfected with F and DSP p1,7 were detached with medium containing EnduRen (Promega), and each was transferred to a separate tube. DSP p8,11-transfected cells were similarly detached and pooled into a single tube. Next, 500 μl of DSP p8,11 cells was added to each tube of F/DSP p1,7 cells, mixed, and plated in medium without BMS-433771 in an oaque 96-well plate (4 wells each at 100 μl/well). Luciferase activity was measured at 2, 4, 6, and 8 h postplating, using a luminometer (PerkinElmer), in quadruplicate. In separate HEp-2 cells, the F plasmids were transfected, and cell surface levels were quantitated 24 h later by cell ELISA as previously described (3), in triplicate. The mean values of F surface expression relative to that of wild-type F protein (Fwt) were used to normalize the luciferase values 6 h postplating. Statistical analysis (Student’s t test, 95% confidence interval) was performed with Graphpad Prism 6.0b.

**IF microscopy.** Infected or transfected cells plated on poly-1-lysine-coated glass coverslips were fixed with freshly dissolved 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 (for N, G, and F proteins) or 0.1% SDS (for M protein), and incubated with anti-N, -M, -G, or -F antibodies. Following incubation with secondary antibodies carrying fluorescent Alexa dyes, cells were stained with DAPI (4',6-diamidino-2-phenylindole), washed, and mounted. Images were captured at 600× magnification on a Nikon TE2000 inverted fluorescence microscope with a DS-Qi1 camera head and DS-U2 camera controller and the NIS-Elements Basic software package (Nikon) or captured at an ~1,500× magnification using a Leica TCS SP2 laser-scanning confocal microscope and processed using Adobe Photoshop CS5.1. Confocal images represent maximum projections of the z-stack images, except for panels 1 and 2 of Fig. 7, for which a single 3-μm slice is displayed to show the internal and perimeter IB association.

**RESULTS**

**Generation of a virus lacking F protein expression.** We previously reported the construction of a virus lacking the entire F protein ORF (38), in which artificially introduced restriction sites were present to facilitate glycoprotein exchange. For this study, a second-generation F-null virus was engineered that lacks any foreign sequences other than the intended changes of the F gene. Using standard molecular biology techniques, the F ORF was removed from an HRSV cDNA (A2 strain) and replaced with an ORF encoding GFP. The resulting cDNA thus contained an exact replacement of the F ORF with that of GFP (Fig. 1A). Infectious virus was recovered from the above cDNA, amplified in baculovirus GP64-expressing Vero cells (Vbac) as previously described (2, 3), and termed RSV-FnullGFP. A wt virus still containing the authentic F ORF was similarly recovered from cDNA to serve as a control virus and termed RSV-WT (Fig. 1A). Virus stocks used for the experiments described were verified by reverse transcription-PCR on RNA harvested from infected cells at passage 3, followed by sequence analysis as previously described (2, 43). The absence of F expression in cells infected by RSV-FnullGFP was verified by cell ELISA (Fig. 1B). HEp-2 cells were infected with RSV-FnullGFP or RSV-WT, and the amounts of N, M, G, and F protein were determined after fixation and permeabilization of infected cells at 24 hpi. Whereas the N, M, and G protein expression levels were similar in cells infected by the two viruses, F protein was detected only in RSV-WT-infected cells, in agreement with the RSV-FnullGFP genome content. Absence of F expression was also observed by IF microscopy (shown below).

**Development of a transient assay to examine F protein CT function.** Using an infectious virus lacking the F protein CT, we previously demonstrated the importance of this domain in production of infectious progeny (3). To allow for a rapid, systematic analysis of all CT residues in a complete virus context, a transient assay based on the F-null virus generated above was developed (Fig. 2A). In this assay, HEp-2 cells are transfected with codon-optimized plasmids expressing the wt F protein (Fwt), a mutant F protein, or no F protein (mock, empty parent plasmid) and subsequently infected with RSV-FnullGFP at 1 PFU/cell. At 34 hpi, cells are scraped into the medium and suspensions frozen at −80°C to capture the total amount of virus produced as previously described (2). Samples are then used to infect “receiver” HEp-2 cells. At 24 hpi, receiver cells are trypsinized and fixed, and the percentage of GFP-expressing cells is counted by flow cytometry to determine the relative amount of infectious progeny virus produced (Fig. 2A). In parallel plates transfected identically, the level of F protein is quantitated by cell ELISA (3) to independently verify F expression.

To test the ability to detect various levels of complementation,
we performed an experiment whereby HEp-2 cells were transfected with increasing amounts of Fwt-expressing plasmid or a mock plasmid as a control. The amounts of infectious progeny virus as well as F expression levels were determined as described above (Fig. 2B). In the absence of F (mock), negligible amounts of infectious progeny were detected, in agreement with the essential nature of the F protein (3). Transfection with increasing amounts of Fwt-expressing plasmid correlated with increasing levels of both F protein (Fig. 2B, right y axis) and infectious progeny virus (Fig. 2B, left y axis), showing that transiently expressed F protein was able to complement RSV-FnullGFP in a dose-dependent manner. Previous studies with a virus expressing an F protein lacking CT amino acids 554 to 574 (FΔCT) demonstrated that the CT is important for production of infectious progeny (3). To examine this under conditions of the F-null-based assay, a plasmid expressing FΔCT was compared to one encoding Fwt (Fig. 2C). Complementation of RSV-FnullGFP-infected cells with Fwt protein led to GFP expression in ∼40% of receiver cells. In contrast, complementation of infected cells with FΔCT resulted in 0.25% of receiver cells expressing GFP, indicating a near-complete lack of infectious progeny production in the absence of the CT. These results show that removal of the CT domain severely curtails infectious virus production in the F-null virus-based assay, in agreement with our previous findings using a CT deletion virus (3).

The relative importance of CT residues for infectious progeny production. To systematically analyze the CT domain, two overlapping series of plasmids were constructed expressing F proteins with double alanine substitutions (Fig. 3A). In case of native alanine residues, the alanines were replaced with glycine. F protein expression by each of the mutants in transfected cells was measured by cell ELISA in the absence (surface F) or presence (total F) of detergent permeabilization (Fig. 3B).

Whereas some substitutions (especially those near the carboxy terminus) resulted in moderately higher F levels, total and surface expression levels of F mutants were similar to that of Fwt protein, indicating that amino acid substitutions in the CT did not substantially lower F expression or block surface transport. To assess the effect of CT mutations on infectious virus production, each series of alanine substitution mutants was tested using the complementation assay described above (Fig. 2A). The results of the two series are shown combined in Fig. 4. A large number of CT substitutions negatively impacted the level of infectious progeny. The results identified an area in the middle of the CT domain with a moderate to strong impact on infectious progeny production (amino acids 557 to 566), termed region 1, or CT-R1, and a smaller area with a very high impact near the carboxy terminus (amino acids 569 to 572), termed region 2, or CT-R2 (Fig. 4A). Several substitutions in CT-R2, which included an amino acid (F572) previously reported to be essential for filament formation (4), completely abolished infectivity, whereas those in CT-R1 reduced infectivity by 40 to 85%. Double substitutions in the membrane-proximal part of the CT had little impact, except for the C550A-K551A double mutant, which reduced infectivity by ∼70%. In spite of the impact of this double substitution, C550 and
mutants were then subjected to the complementation assay. Overall, single alanine substitutions in CT-R1 reduced infectious progeny production to a lesser degree than double substitutions in the same area, and glutamic acid substitution K561E reduced infectivity to lower levels (−25% relative to wt) than did K561A (−50% relative to wt). In CT-R2, all single substitutions had a major impact: I570A and F572A each completely abrogated infectious virus production, and A571G reduced infectivity by 90%. In contrast, replacement of A571 with glutamic acid (A571E) instead of glycine allowed infectious virus production at near-wt levels, indicating that A571 is functionally important but not essential. Based on the above results, amino acids 569 to 572 constituted the region most critical for generation of infectious progeny, even though substitution mutation A571E was well tolerated.

**Substitutions in CT region 1 (amino acids 557 to 566) enhance F-induced membrane fusion.** We previously showed that a virus carrying a deletion of the CT resulted in a strong increase in the level of F-induced syncytium formation (3), which was one of the potential causes of the failure to generate infectious virus in the absence of the F CT. To determine if the CT regions identified above were involved in regulating membrane fusion function, F mutants with changes in CT-R1 (K561A-D562A), CT-R2 (N569A-I570A), or an area in which amino acid substitution did not impact infectious progeny (R553A-S554A) were subjected to a split luciferase-GFP cell-cell fusion assay (41). The C550A-K551A mutant, which significantly impacted infectivity but lay in a region of generally low impact (Fig. 4), was also tested. In short, 293T cells were transfected with an F mutant plasmid and a plasmid expressing a portion of luciferase-GFP. Other cells were transfected with a plasmid expressing the complementing portion of luciferase-GFP. Cells expressing F were incubated in the presence of fusion inhibitor until the time of assay. At 24 h posttransfection, cells were detached, mixed, and plated, and luciferase activity was measured at 2-h intervals. Luciferase activity was normalized by the relative level of F surface expression measured by cell ELISA (Fig. 5). A strong increase in cell-cell fusion was observed in the complete absence of the CT, in agreement with previous work in a viral context (3). We observed no increases for the C550A-K551A and R553A-S554A CT mutants and a moderate increase (−2-fold) for the N569A-I570A mutant. In contrast, a relatively large increase (4.7-fold) was measured after substitution of residues K561 and D562 (CT-R1). These data thus show that deletion of the CT enhances membrane fusion function both in the presence (3) and in the absence of virus and identify CT-R1 as the region largely responsible. The increase in membrane fusion by CT-R1 mutants may be responsible for their poor ability to support infectious virus production. By extension, the lack of impact by substitutions C550A and K551A and moderate impact by N569A and I570A suggests that another mechanism or mechanisms are responsible for the decrease in infectious progeny after substitutions within these areas.

**The F protein CT facilitates viral filament formation.** Another potential mechanism underlying the role of the F protein CT in infectious virus production involves its contribution to viral filament formation. Previous work suggested that the F protein CT is required for viral filament formation (3, 4). In a virus-free assay, it was recently shown that replacement of the CT with the three carboxy-terminal residues F572-S573-N574 was sufficient to allow filament formation (4), albeit at a level about 10-fold lower than that seen with unmutated F protein. This suggests that

K51 appeared to be individually dispensable, since overlapping substitutions with the neighboring adjacent amino acid (Y549A-C550A and K551A-A552G) did not decrease progeny production.

Based on the above findings, a number of mutants carrying single amino acid substitutions in impact and nonimpact regions were generated to examine the effect of individual amino acids on infectivity (Fig. 4B). In some cases, native amino acids were replaced with a charged amino acid (glutamic acid) instead of alanine. F expression levels of the single-amino-acid mutants were verified by cell ELISA as described above (not shown), and the
addition factors, such as spatial requirements or other CT residues, contribute to the efficiency of this process. To examine the impact of the various CT mutations on viral filament formation and to gain insight into the underlying mechanisms, we first tested whether the absence of the CT in an otherwise complete viral context would block viral filament formation. A second F-null virus was generated for this purpose, expressing the blue fluorescent protein (BFP) instead of GFP, to allow optimal use of available fluorophores. This virus was verified as described for RSV-FnullGFP (data not shown) and termed RSV-FnullBFP. To identify viral filaments, both anti-F and anti-M antibodies (and in some cases, anti-N and -G antibodies) were used. This was done because in previous work, generic plasma membrane protrusions and viral surface filaments could be distinguished by the absence or presence, respectively, of additional viral structural proteins, such as M (4). HEp-2 cells were transfected with a plasmid expressing Fwt or FΔCT protein, or a mock plasmid and subsequently infected with virus RSV-FnullBFP. At 24 hpi, cells were fixed, permeabilized to allow simultaneous detection of F and M, and prepared for IF microscopy (Fig. 6). The experiment focused on cells that were successfully transfected and infected (identified by the copresence of F and M) but also yielded individual cells that were transfected but not infected (identified by the presence of F alone) or infected but not transfected (identified by the presence of M but not F).

Cells that received both Fwt plasmid and RSV-FnullBFP carried an abundance of surface filaments to which both the M and F proteins targeted (Fig. 6, panel 1). The G protein colocalized with the M and F proteins in these filaments, as previously reported for a wt virus (data not shown). In the majority of cells that received FΔCT plasmid and RSV-FnullBFP, FΔCT was abundantly expressed, but filaments were not detected (Fig. 6, panel 2). In the majority of mock-transfected cells, filaments were also absent (Fig. 6, panel 3). These findings are in agreement with previous reports and confirm the important role of the F protein CT in filament formation. However, in an estimated 5% of infected cells that expressed FΔCT, labeling with anti-M antibodies identified filament-like structures, which were often clearly protruding from the cell surface (Fig. 6, panel 4). These M-containing structures were also observed (in the same proportion) in cells that received RSV-FnullBFP and mock plasmid (Fig. 6, panel 5), indicating they...
FIG 5 Impact of alanine substitutions in the F protein CT domain on F-induced membrane fusion. Plasmids encoding the indicated F CT substitution mutants, Fwt, or FΔCT were subjected to a dual split-protein assay, in which separately transfected cells each express a portion of a luciferase-GFP fusion protein (41). After transfection, cells expressing F proteins were incubated in medium containing fusion inhibitor. At 24 h posttransfection, cells were detached, mixed, and plated in medium without fusion inhibitor. Luciferase activity was quantitated at various times postplating. Bars represent luciferase activity at 6 h postplating, normalized for F surface expression levels, measured in triplicate by cell ELISA (see Materials and Methods). The data are from one of two replicate experiments performed in quadruplicate and are shown as means ± standard deviations (SD). P < 0.05 (Student’s t test).

formed independently of the F protein. Because the amount of filaments per cell can vary even in a wt virus-infected sample, the amounts of F-independent filaments were difficult to quantitate. However, the average number of F-independent filaments per cell was substantially lower than the number of filaments per cell in Fwt samples, except for a minority of cells (an estimated 5%), in which they were abundant. The number of cells carrying F-independent filaments increased at later times postinfection, with ~20 to 40% of mock- or FΔCT-transfected cells displaying filaments at 36 hpi (data not shown). When FΔCT was present, it was occasionally associated with the filament-like structures, but in the vast majority of cases, it did not locate to the filaments or concentrate in them (Fig. 6, panel 4). To examine if other viral proteins colocalized with the M protein in the F-independent filament-like structures, cells infected with RSV-FnullBFP and left untransfected were costained with anti-M and -G or anti-M and -N antibodies (Fig. 6, panels 6 and 7, respectively). Although minor amounts of G were occasionally observed colocalizing with M in the filament-like structures, G did not concentrate there (Fig. 6, panels 6A, B, and C). In contrast to G, virtually all filaments detected with anti-M antibodies also contained the N protein (Fig. 6, panels 7A, B, and C), suggesting that they represent authentic viral filaments. Thus, despite the important role of the F protein CT, a moderate number of viral filaments formed at the cell surface in an F-independent manner.

In the absence of the F CT, the M and F proteins accumulate in and around IBs. In addition to the noted decrease in viral filament formation, we observed changes in the distribution of the M and F proteins when the F CT was absent. In cells that received RSV-FnullBFP and Fwt at 24 hpi, the M protein was predominantly present in surface filaments and cytoplasm (Fig. 6, panel 1), consistent with a previous report (8) and our observations in wt virus-infected cells. Whereas N is very abundant in IBs throughout the infection cycle (not shown), only small to moderate amounts of M were occasionally detected in these structures. In contrast, upon deletion of the CT or absence of the F protein, we observed an unusually high proportion and high frequency of M protein in IBs (Fig. 6, panels 2 to 6). The M protein was present in IBs that varied in size (Fig. 6, panels 2 to 6) and also contained the N protein (not shown). Increased association of M with IBs occurred both in the absence of the entire F protein and in the absence of the F protein CT, indicating that the F CT was the domain responsible. In addition to changes in M, the absence of the F CT appeared to also induce changes in the distribution of the F protein itself, with a higher proportion of F associated with IBs (Fig. 6, panels 2 and 4). Although an association between F protein and IBs has not been reported in the literature, F was previously observed in filaments that appeared to radiate away from IBs (30). In wt virus-infected cells, we have occasionally observed minor amounts of F protein around IBs by IF microscopy (unpublished observations). However, the level of F surrounding the IBs was very low and constituted a very small proportion of the total F present; hence, the significance of this observation remained unclear. To examine CT-induced F targeting changes in more detail and identify the CT regions potentially involved, we transfected HEp-2 cells with plasmids representing CT-R1 (K561A-D562A), CT-R2 (N569A-I570A), or a region with no impact on infectious progeny (R553A-S554A), and compared them to a parallel sample transfected with FΔCT. The C550A-K551A substitution mutant was also tested. Following transfection, cells were infected with RSV-FnullBFP and examined by confocal microscopy 24 hpi after staining with anti-F and anti-M antibodies (Fig. 7). Optical sectioning and analysis of 3-μm sections revealed that in the presence of FΔCT, the M protein accumulated inside the IBs, whereas a noticeable portion of F accumulated around IBs, sometimes in a patchy fashion (Fig. 7, panels 1A, B, and C). This phenotype was highly consistent and repeatable; moreover, virtually every IB in cells expressing FΔCT protein displayed an increased presence of F at its perimeter relative to Fwt. The unusual accumulation of F around IBs also occurred in the presence of the N569A-I570A F substitution mutant (CT-R2) (Fig. 7, panels 2A, B, and C). In contrast, in the presence of all other tested F mutants, M and F were not present in or near IBs but instead localized to viral surface filaments (Fig. 7, panels 3 to 5), thus identifying CT region 2 as the responsible region. To exclude that this was a HEp-2 cell-type-specific phenomenon, selected constructs were similarly analyzed in Vero cells using epi-fluorescence, with identical results (Fig. 7, panels 6 to 8). The strong reduction in filaments and simultaneous accumulation of M and F in and around IBs upon CT deletion or CT-R2 mutation suggests that the F protein targets to the IB perimeter under wt conditions (independent of the CT domain) to mediate a step in the transition of assembly complexes from IBs to filaments for which CT region 2 is required.

DISCUSSION

The mechanisms by which the F protein CT facilitates infectious virus production, including an anticipated interaction with the M protein, have been elusive. We developed an F-null virus-based flow cytometry assay to screen the effects of F CT mutations on infectious virus production as a starting point to discover the mechanisms involved. This assay is both rapid and safe (does not involve the production of engineered viruses) yet comprehensive
FIG 6 Impact of F protein CT deletion and F absence on viral protein distribution and viral filament formation. HEp-2 cells were transfected with plasmids encoding Fwt (panel 1) or FΔCT (panels 2 and 4) or a mock plasmid (panels 3 and 5) or were left untransfected (panels 6 and 7). Following transfection, all samples were infected with RSV-FnullBFP. At 24 hpi, cells were fixed with 4% paraformaldehyde, permeabilized, and incubated with anti-N, -M, -G, or -F antibodies. Following incubation with Alexa-conjugated secondary antibodies, cells were stained with DAPI and mounted, and images were captured at ~1,500× magnification on a laser-scanning confocal microscope. The plasmids used are indicated in the left upper corner of each panel. The primary antibodies used and the color of the secondary conjugate are indicated in the upper right corners. Viral surface filaments and IBs are indicated with white arrows and arrowheads, respectively. Insets represent a higher-magnification detail of the regions marked by an asterisk. The difference between panels 2 and 4 and panels 3 and 5 is that panels 2 and 3 represent the majority phenotype (lack of viral filaments), whereas panels 4 and 5 represent the minority phenotype (presence of F-independent viral filaments). Note that as a result of the transfection/infection protocol, some pictures contain additional cells that were transfected but not infected (presence of F alone) or infected but not transfected (presence of N, M, or G but not F). All images represent maximum projections (merge of all z-stacks).
FIG 7 Effect of F protein CT mutations on viral protein distribution. HEp-2 cells were transfected with plasmids encoding F lacking the CT (FΔCT, panels 1A, B, and C) or the mutants N569A/I570A (panels 2A, B, and C), C550A/K551A (panel 3), R553A/S554A (panel 4), and K561A/D562A (panel 5) and subsequently infected with RSV-FnullBFP. At 24 hpi, cells were fixed with 4% paraformaldehyde, permeabilized, and incubated with anti-M and -F antibodies. Following incubation with Alexa-conjugated secondary antibodies, cells were stained with DAPI and mounted, and images were captured on a laser-scanning confocal microscope at ~1,500× magnification. In panels 6 to 8, Vero cells were similarly transfected with plasmids encoding Fwt (panel 6), FΔCT (panel 7), or mutant N569A/I570A (panel 8), infected with RSV-FnullBFP, and analyzed on an epifluorescence microscope at 600× magnification. The plasmids used are indicated in the left upper corner of the panel. The primary antibodies used and the color of the secondary conjugate are indicated in the upper right corners. White arrows and arrowheads identify the cell analyzed and respectively indicate viral surface filaments and IBs. Insets represent a higher-magnification detail of the regions marked by an asterisk. Note that as a result of the transfection/infection protocol, some panels contain additional cells that were transfected but not infected (presence of F alone) or infected but not transfected (presence of M but not F). Panels 3, 4, and 5 represent maximum projections (merge of all z-stacks); panels 1 and 2 represent a single 3-μm optical section to show the association of F with IBs.
MECHANISMS UNDERLYING F CT FUNCTION.

The mechanism of filamentous virion formation remains poorly understood. Our data shed light on this mechanism, detailing F protein CT-dependent changes in association of M and F with cytoplasmic IBs. Although M was reported to accumulate in IBs starting at 14 hpi (36), we typically find low steady-state levels of M in IBs under wt conditions throughout the infection cycle. When filaments become abundant, the proportion of M associated with IBs is very low, and most of the M protein is associated with filaments and the cytoplasm. Based on the low levels of M in IBs under wt conditions, we suspect that the association of M with IBs—and the same appears to be the case for association of F with the IB perimeter—is short-lived. This may be due either to rapid release of assembly complexes once M and F have arrived or rapid M- and F-induced progression or structural disintegration of the IBs in preparation for filament formation. In contrast, under FΔCT and ΔF conditions and upon substitutions in CT-R2, M remained relatively abundant in the cytoplasm but concentrated in IBs of various sizes. In addition, increased amounts of the F protein surrounded the IBs in the absence of the CT. This change in M and F targeting coincided with a strong decrease in viral surface filaments, indicating that interactions of both M and F with IBs are an important step in the process of assembly, preceding the formation of filamentous virions. The latter lends support to previous propositions that the IBs may constitute a type of scaffold from which viral filaments are formed or initiated (2, 18, 30, 31). F accumulation around IBs also suggests that the IBs are surrounded by a membrane, in support of a previous electron microscopy (EM)-based observation of a membrane-like layer near the IB edge (30). It is noteworthy that despite accumulation around IBs, F remained relatively abundant at other cellular sites (such as cell surface and internal vesicle-like structures), leaving open the possibility that different populations of F might exist with different roles in the infection cycle. The continued presence of M in the cytoplasm upon CT deletion and coincident decrease in filament formation suggest that cytoplasmic M not associated with IBs is not readily incorporated into filaments, also supportive of a mechanism in which M must transiently associate with IBs prior to initiation of filament formation. In this proposed scheme, CT-R2 amino acids might function to facilitate release of immature assembly complexes from the IB, by direct or indirect interaction with M or by inducing structural changes that support the role of M. Whether or not M and F interact during the assembly process, it is clear that F and M targeting to the IBs does not require the F CT.

CT AMINO ACIDS OUTSIDE REGION 2.

Substitutions outside CT-R2 did not lead to accumulation of M in IBs or abnormal filament formation. Therefore, the negative impact of CT-R1 or 550/551 alanine substitutions on infectious progeny production is probably unrelated to the assembly mechanism. In case of CT-R1, improper fusion function may underlie the negative impact on infectious progeny as substitution of residues 561 and 562 resulted in enhanced cell-cell fusion in a split-luciferase assay. Although the kinetics and mechanisms of cell-cell fusion may differ from those of virus-cell fusion, it is, by extension, possible that CT-R1 is involved in prevention of premature fusion during assembly and maturation or in regulation of proper virus-cell fusion activity or triggering during viral entry. Although the CT is in the cytoplasmic compartment and relatively distant from the ectodomain, other studies have suggested that CTs and TMDs can influence membrane fusion activity through impact on protein ectodomain conformation (41, 44). Transient expression of F lacking the entire CT strongly enhanced cell-cell fusion (Fig. 5), and this was also the case when FΔCT was expressed in a virus context (3). Since the enhanced fusion occurred in both the presence and absence of virus, it is probably not caused by interference of any putative M-F interaction. A previous study using transiently expressed F protein lacking the CT reported moderately lower levels of membrane fusion (45). In that case, however, amino acids 549 to 574 were removed, leaving no positively charged amino acids following the TMD, which may have resulted in inappropriate membrane anchoring of F (46). In that regard, C550 and K551 each have potential to contribute to F protein membrane anchoring: C550 via its attached palmitate group (47) and K551 by providing a positive charge directly following the TMD (46). The latter would be consistent with a previous finding in which deletion of the CT, including K551, led to perinuclear targeting and aberrant colocalization of F with calnexin in the endoplasmic reticulum, whereas deletion of the CT beginning after K551 resulted in transport of F to the plasma membrane (48).

THE F PROTEIN CT IS IMPORTANT BUT NOT ESSENTIAL FOR FILAMENT FORMATION.

Although the F protein CT had a profound impact on the number of viral filaments, in agreement with previous reports (3, 4), a moderate number of F-independent viral filaments formed, especially at later times postinfection. Because these filaments contained the M and N proteins, they appear to constitute genuine viral filaments and not cellular protrusions (4). A continued (low-level) production of filamentous virions lacking G would be in agreement with a previous report, in which viral particle production in A549 cells was not blocked entirely in the absence of F and in which the level of M-N association was unchanged, whereas the level of G-N association was decreased (39).
However, whether the F-independent filament formation represents a minor in vivo assembly pathway or an artifact only occurring in the absence of F or FΔCT, remains to be seen. F-independent filaments were not reported in a recent study coexpressing F variants with N, P, and M in the absence of viral infection (4), indicating that additional viral proteins may be required. Alternatively, filament formation in the absence of viral infection may have been less robust and insufficient to allow development of F-independent filaments. The F-independent filaments formed under conditions where M accumulated in IBs. This may have occurred by a mechanism that is independent of IBs and thus mediated by free M protein (i.e., cytoplasmic but not associated with IBs). This appears feasible given the intrinsic property of M to self-assemble into helical arrays in the presence of select lipid mixtures (49). However, if IB independent, it is unclear how RNPs would be recruited to filaments since genomic RNA has been linked to IBs (29, 33). If the F-independent filaments do not contain viral RNA, it is similarly unclear how N protein would be incorporated into these filaments. Alternatively, our data may indicate that the filament formation process is in essence a slow M-driven process and that the F protein CT acts as a facilitator. The absence of F or the F CT then would merely slow down the progression of assembly complexes from IBs, resulting in gradual accumulation of M (and FΔCT if present) at IBs and an ongoing low-level filament formation.

In conclusion: a model for the role of the F protein CT in filament assembly. One interpretation of our combined findings suggests a model in which the F CT acts as a type of catalyst in an M-driven filament formation process that depends on relatively short-lived association of M and F with IBs. In this scenario, M binds to encapsidated viral RNA within IBs and F targets to IBs independent of the CT to engage assembly complexes at the periphery. It remains unclear whether F and M interact during this process. In the presence of, and facilitated by, the F protein CT, assembly complexes are then released to be incorporated into filaments or to proceed rapidly to sites of filament production near the plasma membrane using host machinery. Even though the F CT may act as a catalyst, this process can be driven by M alone, as filaments continued to form at a low rate in the absence of the F CT and in the absence of F entirely. The major purpose of the F CT in this model then would be to accelerate virus dissemination by ensuring rapid filamentous virion formation, with CT amino acids 569 to 572 constituting the key functional residues. A catalytic function of the F CT in filament formation would also ensure that most filaments contain the essential F protein. If F failed to incorporate into filaments during their formation, the CT could also serve to retroactively target F protein to ensure fusogenic capacity. In short, in addition to potential roles in membrane anchoring and membrane fusion, the F CT may both facilitate the process of viral filament formation and serve to incorporate F protein into existing filaments.

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REFERENCES