Mobility and Interactions of Coronavirus Nonstructural Protein 4^{▽†}

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Green fluorescent protein (GFP)-tagged mouse hepatitis coronavirus nonstructural protein 4 (nsp4) was shown to localize to the endoplasmic reticulum (ER) and to be recruited to the coronavirus replicative structures. Fluorescence loss in photobleaching and fluorescence recovery after photobleaching experiments demonstrated that while the membranes of the ER are continuous with those harboring the replicative structures, the mobility of nsp4 at the latter structures is relatively restricted. In agreement with that observation, nsp4 was shown to be engaged in homotypic and heterotypic interactions, the latter with nsp3 and nsp6. In addition, the coexpression of nsp4 with nsp3 affected the subcellular localization of the two proteins.

All positive-strand RNA viruses induce modified cellular membranes in infected cells, onto which their replication complexes are anchored (reviewed in references 23 and 30). Coronaviruses induce the formation of double-membrane vesicles (DMVs) and convoluted membranes (CMs) (14, 18, 24, 29, 32, 34), which form a large reticulovesicular network with which viral replication and transcription are associated (14, 18, 37). The nonstructural proteins (nsp's) localize to the CMs and the DMVs (16, 18, 34, 37), while double-stranded RNA (dsRNA), probably corresponding to replicative intermediates, has been detected at the DMV interior (18, 34). Not much is known about the mechanism by which the coronavirus replicative structures (DMVs and CMs) are formed (4, 26), let alone about the functioning of the different structures in viral RNA synthesis (18, 37).

The coronavirus nsp's 3, 4, and 6 are integral membrane proteins. These proteins, the membrane topology of which was recently established (1, 17, 24, 25), are supposed to drive the induction of the membrane rearrangements and to provide the scaffold onto which the replication complexes are assembled. Key roles have been attributed in particular to nsp3 and nsp4 in the induction of the typical membrane structures, as ectopic coexpression of the arterivirus nsp3 and nsp4 counterparts results in the appearance of the corresponding structures (35). The importance of coronavirus nsp4 in DMV biogenesis is indicated by several other observations. nsp4 was shown to be an essential protein (36), while mutation of residue 258 resulted in a temperature-sensitive phenotype, with reduced numbers of DMVs and nsp4 localizing to the mitochondria at the restrictive temperature (3). Abrogation of the nsp4 glycosylation sites led to an impairment of viral RNA synthesis

accompanied by the appearance of aberrant DMVs and increased numbers of CMs (13).

In view of the essential role of nsp4 in replication and the assembly of the replicative structures, we analyzed its dynamics using fluorescence loss in photobleaching (FLIP) and fluorescence recovery after photobleaching (FRAP) approaches. As we have been unable to generate recombinant viruses expressing green fluorescent protein (GFP)-tagged nsp4 in the context of the replicase precursor proteins, we used a recombinant mouse hepatitis virus (MHV) in which a GFP-tagged version of nsp4 was expressed as an additional expression cassette (MHV-nsp4GFP) (25).

MHV-nsp4GFP replication kinetics and peak titers in LR7 cells were similar to those of recombinant wild-type MHV (MHV-WT) (5) (Fig. 1A). Apparently, expression of nsp4-GFP, which was expressed approximately 12-fold more abundantly than the endogenous mature nsp4 protein (data not shown), did not interfere with virus replication. Consistent with our earlier observations (25), the protein displayed a reticular staining pattern that coincided with the endoplasmic reticulum (ER) marker calreticulin (Fig. 1B). In addition, nsp4-GFP was also present in puncta that colocalized not with calreticulin but with markers for the coronavirus replicative structures, such as nsp8 and nsp2/3 (Fig. 1B) (25). Most nsp4-GFP-positive foci colocalized with or were found adjacent to newly synthesized viral RNA (Fig. 1C), which was detected by feeding cells with an alkyne-modified nucleoside, 5-ethynyl uridine (EU), as described previously (39), indicating that nsp4-GFP is recruited to sites of active RNA synthesis. Finally, we studied the localization of nsp4-GFP by analyzing MHV-nsp4GFP-infected cells by immunoelectron microscopy (IEM) using anti-GFP antibodies as described previously (16, 33, 37, 38). Although the labeling was weak, it was specific and showed nsp4-GFP localization on the surface of the DMVs, which appeared as empty holes inherent to the method used (Fig. 1D) (16, 37, 39). Some additional staining could be observed on membranes that probably correspond to either the CMs or the ER. No labeling of noninfected cells was observed (data not shown).

The presence of nsp4-GFP in ER membranes and its recruitment to the viral replicative structures allowed us to assess

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FIG. 1. Characterization of recombinant MHV-nsp4GFP virus. (A) LR7 cells were infected with either MHV-nsp4GFP or recombinant wild-type MHV (MHV-WT; 5) at a multiplicity of infection (MOI) of 10. Culture media were collected at the indicated time points postinfection (p.i.), and viral infectivity was determined by performing quantal assays with LR7 cells. TCID50, 50% tissue culture infective dose. (B) LR7 cells were infected with MHV-nsp4GFP, fixed, and subjected to immunofluorescence analysis using antibodies directed against calreticulin (Sigma) and nsp2/3 (31). (C) The sites of viral RNA synthesis in MHV-nsp4GFP-infected cells were visualized by EU labeling at 6 h p.i., followed by fixation and coupling of an Alexa 594 fluorophore to the incorporated EU using click chemistry (Click-iT; Invitrogen). (D) Cryosections of MHV-nsp4GFP-infected cells were prepared and incubated with antibodies directed against the GFP tag (Abcam), followed by immunogold labeling (indicated by the arrowheads) (33). The asterisk indicates the ER and/or the convoluted membrane assemblies. Bar, 200 nm.

whether the membranes of the DMVs/CMs and the ER are interconnected in MHV-infected cells. We applied FLIP to MHV-nsp4GFP-infected cells to verify whether this continuity existed. With this technique, a specific area of the cell is repeatedly photobleached, and loss of fluorescence outside the bleached area is monitored (for more details, see references 19 and 20). After a specific area has been photobleached, fluorescence is recovered by diffusion. When the same area gets repeatedly bleached, the fluorescence of the whole organelle will be lost. Thus, while loss of fluorescence outside the photobleached area is indicative of membrane continuity, a persisting signal indicates the lack of connectivity between membrane systems. At 6 h p.i., a defined region targeting the ER in MHV-nsp4GFP-infected cells was repeatedly photobleached, each time followed by the acquisition of postbleaching images at different locations in the cell. No fluorescence loss was observed for neighboring cells. Repeated photobleaching of infected cells resulted in fluorescence loss of the nsp4-GFP present in the ER as well as in the replicative structures (Fig. 2A and B) (see Video S1 in the supplemental material). However, significantly less fluorescence was lost after repeated photobleaching over a period of 30 min in the replicative struc-

tures than that lost in the ER (60% versus 80%). Furthermore, nsp4-GFP fluorescence decreased significantly faster in the ER than in the replicative structures ($P = 0.011$).

These results revealed continuity between the ER and the replicative structures. Although we cannot exclude the possibility that this continuity results from (rapid) vesicular transport between these structures, direct continuity between the membranes of the ER and of the replicative structures appears a more likely explanation in view of an electron tomography study of severe acute respiratory syndrome coronavirus (SARS-CoV)-infected cells (18). For MHV-A59, electron tomography may be required to demonstrate this kind of continuity, as it was previously not observed for MHV-infected cells by conventional electron microscopy (37). The electron tomography study of SARS-CoV-infected cells also showed that the inner membranes of the DMVs are sealed and do not display continuity with the other membranes. The absence of any "pockets" of fluorescence, i.e., regions of restricted mobility, in our FLIP experiments indicates either that nsp4 is present at minimal levels in the inner lipid bilayer of the DMVs or that continuity between the inner and the outer membranes exists.

The FLIP experiments indicated that the nsp4-GFP proteins

FIG. 2. FLIP and FRAP analyses of MHV-nsp4GFP-infected cells. (A) In the FLIP experiments, the ER of MHV-nsp4GFP-infected cells was repeatedly photobleached, 25 times for 1 s every 60 s, and fluorescence loss was monitored at the nsp4GFP-positive replicative structures (dots) or at sites that contain reticular nsp4-GFP fluorescence. Representative images of such an experiment are depicted and were enhanced using the “hot” look-up table of ImageJ (28), in which the observed fluorescence is represented as colors corresponding to the strength of the fluorescence intensities, ranging from white/yellow (high intensities) to purple/black (low intensities). The bleached area is indicated by the white box. t, time. (B) The recovery fractions of the FLIP experiments were obtained by background correction of the measured fluorescence intensities and by normalizing these to the prebleach fluorescence intensities at the indicated cellular locations. The mean results from five experiments are shown. The error bars indicate the standard errors of the means. (C) FRAP was performed as previously described (16), using MHV-nsp4GFP-infected cells, thereby targeting nsp4-GFP-positive dots or sites containing reticular nsp4-GFP fluorescence only. The corresponding fluorescence recovery graphs, after background correction and normalization of the measured fluorescence intensities, are depicted, and calculated mobile fractions (MF) are indicated. The mean results from six and nine experiments are shown for nsp4-GFP-positive dots and reticular nsp4-GFP, respectively. The error bars indicate the standard errors of the means. FR, fluorescence recovery; norm factor, normalization factor. (D) Representative images of such FRAP experiments are depicted, with the bleached area indicated by the white box. For all live-cell imaging experiments, the spike protein heptad repeat 2 (HR2) peptide (2) was added to inhibit cell-cell fusion.

display different diffusion properties depending on their sub-cellular localization. To confirm this observation, we investigated the mobility of nsp4-GFP in more detail by performing FRAP experiments, as previously described (for details, see references 16, 20, 21, and 39), targeting the nsp4-GFP fluorescence in MHV-nsp4GFP-infected cells either at the ER (reticular staining) or at the replicative structures (dots); representative images and corresponding fluorescence recovery graphs are depicted in Fig. 2C and D. Bleaching of reticular

nsp4-GFP resulted in a reduction of ~50% of the prebleaching fluorescent signal. Within 30 s, ~35% of the nsp4-GFP fluorescent signal was recovered, with a calculated mobile fraction (Mf) of 64.9%. These results indicate that nsp4-GFP is able to laterally diffuse through the lipid bilayers of the ER, in agreement with our FLIP experiments. Photobleaching of nsp4-GFP-positive dots resulted in a reduction of ~60% of the prebleached signal. Much less recovery of the fluorescent signal was observed at these structures (Mf of 33.1%). Further-

FIG. 3. CoIP and PCA analysis of homo- and heterotypic interactions of nsp4. GFP- and HA-tagged nsp constructs were expressed in OST7-1 cells, either alone or in the following combinations: (A) nsp4-GFP with nsp4-HA, (B) nsp3_C-GFP with nsp4-HA and nsp3_C-HA with nsp4-GFP, and (C) nsp4-GFP with nsp6-HA and nsp4-HA with nsp6-GFP. Cells were radiolabeled for 1 h. Cell lysates were prepared and subjected to IP with either anti-GFP (α -GFP) or anti-HA (α -HA) antibodies (Immunology Consultants Laboratory, Inc.). Precipitates were analyzed using SDS-PAGE. As a control for the coexpression (co), lysates of singly expressed proteins were pooled (pl) and processed similarly for IP. The black and gray arrowheads indicate the positions in the gel of GFP- and HA-tagged proteins, respectively. (D) vTF7-3-infected OST7-1 cells were transfected with the indicated combinations of the split-Venus PCA fragments, after which the cells were fixed at 6 h p.i. and processed for immunofluorescence microscopy. Representative images are shown. (E) The mean arbitrary fluorescence intensities of 10 randomly chosen cells per cotransfection in the PCA experiments were determined using a Deltavision RT microscope and the Velocity software package from Improvision. V1 and V2 indicate Venus fragments 1 and 2.

more, it appeared that the measured recovery resulted largely from mobility of nsp4-GFP in ER membranes, which are also present in the bleached areas, rather than from recovery in the replicative structures.

The FRAP experiments are consistent with the FLIP data and show that while the nsp4-GFP protein pool present at the replicative structures is mobile, its mobility is clearly less than that of the nsp4-GFP present in ER membranes. Apparently, the protein experiences some kind of diffusion barrier when present at the replicative structures. Until now, we have analyzed the mobility of two other proteins at the replicative structures: nsp2 (16) and the nucleocapsid protein N (39). While nsp2, once recruited to the replication-transcription complexes (RTCs), was immobilized (16), the N protein was dynamically associated to the RTCs (39). Although the recovery of nsp4 and nsp2 to the bleached replicative structures cannot be compared directly, as nsp2 is a cytosolic protein and nsp4 is an integral membrane protein, it appears that nsp2 is constrained at the replicative structures to a much greater extent than is nsp4.

As all nsp's studied to date are located at the replicative

structures (8, 15, 25, 27, 31, 34), the local interaction of nsp4-GFP with other nsp's may very well account for the observed differences in diffusion. Therefore, we investigated whether nsp4 binds to other nsp's. We decided to focus on the coronavirus integral membrane nsp's (nsp3, nsp4, and nsp6), as the equine arterivirus (EAV) counterparts of the coronavirus nsp3 and nsp4 have previously been shown to interact with each other (35). The proteins were expressed in OST7-1 cells using the recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase (vTF7-3) expression system (11, 12), after which cells were labeled with ³⁵S-labeled amino acids and coimmunoprecipitation (coIP) experiments were performed as described previously (6, 7). The gene fragments encoding the nsp's were fused to either GFP- or hemagglutinin (HA)-encoding sequences and cloned under the control of a T7 promoter (24, 25). For nsp3, only the fragment encoding the C-terminal domain (nsp3_C), which contains all transmembrane domains, was cloned (24), as the gene encoding the full-length protein appeared to be too toxic to clone and express. Interaction between the coexpressed proteins is monitored by the coprecipitation of HA-tagged proteins using anti-GFP anti-

FIG. 4. Coexpression of nsp4 and nsp3_C results in altered subcellular localization of these proteins. vTF7-3-infected OST7-1 cells were (co)transfected with plasmids encoding, nsp3_C-HA, nsp4-HA, nsp6-HA, nsp3_C-GFP, nsp4-mCherry, nsp4-GFP, or nsp6-GFP as indicated in the figure. Six hours p.i., cells were fixed and processed for confocal microscopy. (A) Single expression. Cells were subjected to immunofluorescence analysis using antibodies directed against the HA tag and calreticulin. α , anti. (B) Coexpression experiments with nsp3_C, nsp4, and nsp6. The arrowhead points to a cell demonstrating perinuclear fluorescent foci as observed in nsp3_C- and nsp4-cotransfected cells, whereas the arrow points to a single transfected cell expressing only reticular nsp3_C-GFP, for which these dots are not observed. (C) Coexpression of nsp3_C-HA and nsp4-GFP also results in the appearance of perinuclear fluorescent foci. The panel shows multiple cells in a field.

bodies and *vice versa*. As a control for the specificity of the detected interactions, lysates of cells singly expressing the nsp's were pooled and subsequently processed similarly for IP, as described previously (6). As an additional control, the coIP assay was also applied after coexpression of nsp4 with the MHV triple-spanning membrane (M) protein. Antibodies directed against the GFP or HA tag of nsp4 did not coprecipitate the MHV M protein, and M protein-specific antibodies did not coprecipitate the nsp4 fusion proteins (data not shown). We first investigated whether nsp4 is engaged in homotypic interactions (Fig. 3A). The pooled lysates of separately expressed nsp4 proteins did not demonstrate coIP of nsp4, demonstrating the specificity of the assay and of the anti-GFP and anti-HA antibodies. When coexpressed, however, nsp4-GFP was coprecipitated with nsp4-HA and *vice versa*. Similar results were obtained when nsp4 fusion proteins were coexpressed with nsp3_C or nsp6 fusion proteins (Fig. 3B and C).

As a second independent assay to detect these interactions, we made use of the yellow fluorescent Venus protein-based protein complementation assay (PCA) (22). In this assay, two complementary Venus reporter fragments, V1 and V2, are fused to the protein(s) of interest. Upon interaction of the fusion proteins, the Venus fluorescent reporter activity is reconstituted. C-terminal V1 and V2 reporter fusion constructs of nsp3_C, nsp4, and nsp6 were generated and coexpressed in different combinations using the vTF7-3 expression system, after which the cells were processed for (quantitative) fluorescence analysis. Cotransfection of the empty V1 or empty V2 plasmid with the nsp4-V2 or nsp4-V1 construct did not result in reconstitution of the Venus fluorescence and served as a negative control (Fig. 3D and E), in agreement with the in-

ability of the Venus reporter fragments to spontaneously re-fold in their native structure in the absence of interacting partners being fused to them (22). With this assay, we were able to confirm the interaction of nsp4 with itself and with nsp6, as a reticular fluorescence signal was observed when these gene fragments were coexpressed (Fig. 3D and E). However, we were not able to demonstrate interaction between nsp4-V1 and nsp3_C-V2, and only occasionally was a weakly positive cell found for the nsp4-V2 and nsp3_C-V1 combination. The absence of fluorescence was not due to the nsp3_C constructs themselves, as coexpression of nsp3_C-V1 and nsp3_C-V2 clearly resulted in reconstitution of fluorescence, indicative of nsp3 homotypic interactions (data not shown). The fact that the interaction between nsp4 and nsp3_C was not observed with PCA may be caused by the C termini of the two proteins not being oriented in a configuration allowing the reconstitution of the fluorescence activity. In agreement with the coIP results, interaction between nsp4 and nsp3 was observed using yeast two-hybrid assays (data not shown). These results show that nsp4 is contained within a network of protein-protein interactions and probably explain the recruitment of nsp4 to the RTCs when expressed in *trans* (reference 25 and this study). Moreover, nsp4 is likely to play a central role within this network, as the infection of cells with a temperature-sensitive MHV, carrying a mutation in nsp4, resulted in a partial localization not only of nsp4 but also of nsp3 and possibly other nsp's to mitochondria at the restrictive temperature (3).

Finally, we analyzed whether coexpression of nsp4 with nsp3_C or nsp6 would affect the localization of the proteins, similarly to what has been described for EAV nsp2 and nsp3 (35). First, we studied the subcellular localization of the C-ter-

minally tagged membrane proteins using the vTF7-3 expression system. As shown in Fig. 4A and in agreement with previous reports (1, 17, 24, 25), individually expressed nsp3_C, nsp4, and nsp6 exhibited a reticular pattern and colocalized with the ER marker calreticulin regardless of the identity of the C-terminal tag (data not shown). Next, we coexpressed nsp3_C, nsp4, and nsp6 in various combinations. While the reticular staining pattern of these proteins by coexpression of nsp6 with either nsp4 or nsp3_C was not affected, coexpression of nsp4 with nsp3_C resulted in the formation of perinuclear fluorescent foci in which these proteins appeared to be concentrated (Fig. 4B). Similar results were obtained when nsp4 or nsp3_C carried different tags (Fig. 4C). We hypothesize that the coexpression of nsp3_C and nsp4 results in a rearrangement of host cell membranes. We are currently studying to what extent these rearrangements resemble the membrane modifications in MHV-infected cells (37). For several other plus-strand RNA viruses, the viral proteins responsible for the membrane rearrangements, with which viral RNA synthesis is associated, have been identified (reviewed in references 9 and 10). These key organizers of plus-strand RNA virus replication complexes appear to have in common the characteristic of exerting their function as oligomeric complexes. In this respect, this study shows that the coronavirus transmembrane-containing nsp's are no exception.

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