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Membrane rearrangements mediated by coronavirus nonstructural proteins 3 and 4

Marne C. Hagemeijer [a,1], Iryna Monastyrska [a], Janice Griffith [b], Peter van der Sluijs [b], Jarno Voortman [c], Paul M. van Bergen en Henegouwen [c], Annelotte M. Vonk [a], Peter J.M. Rottier [a], Fulvio Reggiori [b], Cornelis A.M. de Haan [a,*]

[a] Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands
[b] Department of Cell Biology and Institute of Biomembranes, University Medical Centre Utrecht, Utrecht, The Netherlands
[c] Division of Cell Biology, Department of Biology, Science Faculty, Utrecht University, Utrecht, The Netherlands

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ABSTRACT

Coronaviruses replicate their genomes in association with rearranged cellular membranes. The coronavirus nonstructural integral membrane proteins (nsps) 3, 4 and 6, are key players in the formation of the rearranged membranes. Previously, we demonstrated that nsp3 and nsp4 interact and that their co-expression results in the relocalization of these proteins from the endoplasmic reticulum (ER) into discrete perinuclear foci. We now show that these foci correspond to areas of rearranged ER-derived membranes, which display increased membrane curvature. These structures, which were able to recruit other nsps, were only detected when nsp3 and nsp4 were derived from the same coronavirus species. We propose, based on the analysis of a large number of nsp3 and nsp4 mutants, that interaction between the large luminal loops of these proteins drives the formation of membrane rearrangements, onto which the coronavirus replication–transcription complexes assemble in infected cells.

Introduction

One of the hallmarks of positive-strand RNA (+RNA) viruses is the induction of membranous rearrangements of varying morphologies that serve as platforms for the viral replication and transcription complexes (RTCs) that are intimately associated with them [reviewed in Miller and Krijnse-Locker (2008, den Boon and Ahlquist (2010a), den Boon et al. (2010b), Delang et al. (2012)]. Assembly of the viral RTCs on host-derived membranes provides a dual advantage for the virus by (i) ensuring that cellular and viral constituents required for RNA synthesis are optimally spatio-temporally organized and by (ii) concealing the multiple viral RNA species generated during viral replication from the anti-viral host defense mechanisms of the infected cell. It is becoming apparent that the generation of these replication platforms depends on the concerted actions of hijacked host and viral membrane-shaping proteins, lipid-modifying enzymes and various exploited cellular pathways [reviewed in Miller and Krijnse-Locker (2008), den Boon and Ahlquist (2010a), den Boon et al. (2010b), Altan-Bonnet and Balla (2012), Belov and van Kuppeveld (2012), Delang et al. (2012), Hagemeijer et al. (2012a)].

Coronaviruses (CoVs) are enveloped +RNA viruses that belong to the family *Coronaviridae* in the order of the *Nidovirales*. The CoV-induced replicative structures consist of double-membrane vesicles (DMVs) and convoluted membranes (CMs) (Gosert et al., 2002; Goldsmith et al., 2004; Snijder et al., 2006; Ulasli et al., 2010), which form an interconnected reticulovesicular network of modified membranes that appears to be continuous with the endoplasmic reticulum (ER) (Knoops et al., 2008; Hagemeijer et al., 2011), as has been observed in cells infected with severe acute respiratory syndrome (SARS)-CoV or mouse hepatitis virus (MHV). Similar structures have also been observed for the recently emerged Middle East respiratory syndrome (MERS)-CoV (de Wilde et al., 2013). Recently Maier et al. demonstrated that in addition to DMVs and CMs, the avian CoV infectious bronchitis virus (IBV) induces small double-membrane spherule-like structures that are associated with zippered ER membranes (Maier et al., 2013). These structures have not been observed however in SARS-CoV, MHV- or MERS-CoV-infected cells. Replication-associated viral proteins have been demonstrated to localize both the DMVs and CMs (Shi et al., 1999; van der Meer et al., 1999; Goldsmith et al., 2004; Prentice et al., 2004; Graham et al., 2005; Snijder et al., 2006; Deming et al., 2007; Oostra et al., 2007; Knoops et al., 2008;

* Corresponding author. Tel.: +31 30 2534195; fax: +31 30 2536723.
E-mail address: c.a.m.dehaan@uu.nl (C.A.M. de Haan).
[1] Present address: Laboratory of Host-Pathogen Dynamics, Cell Biology and Physiology Center (CBPC), National Heart Lung and Blood Institute (NHLBI), National Institutes of Health, Bethesda, MD, USA.

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Reggiori et al., 2010; Ulasli et al., 2010) whereas double-stranded RNA (dsRNA) – presumably (once) functioning as replicative intermediate during viral RNA synthesis – has been detected in the interior of the DMVs (Knoops et al., 2008). Early in infection, viral proteins and nascent RNAs colocalize with or occur adjacent to dsRNA puncta, whereas the correlation between these different molecules is less obvious late in infection (Hagemeijer et al., 2012b). It has recently been proposed that CoVs hijack EDEMosomes, ER-derived vesicles involved in the transport of selected short-lived ER chaperones to the endosomal system, to generate the CoV replicative structures (Reggiori et al., 2010; Bernasconi et al., 2012).

When compared to other +RNA viruses, CoVs possess extremely large genomes that range in size from ~26 to 32 kb (Gorbalenya et al., 2006). Two-thirds of the genome encodes the nonstructural proteins (nsps), which together with the nucleocapsid (N) protein, and presumably host proteins, form the membrane-associated RTCs (Shi et al., 1999; van der Meer et al., 1999; Goldsmith et al., 2004; Prentice et al., 2004, Graham et al., 2005; Snijder et al., 2006; Deming et al., 2007; Oostra et al., 2007; Knoops et al., 2008; Reggiori et al., 2010; Ulasli et al., 2010; Verheije et al., 2010). Among the 16 nsps, three contain multiple hydrophobic, membrane-spanning domains: nsp3, nsp4 and nsp6 (Harcourt et al., 2004; Kanjanahaluethai et al., 2007; Oostra et al., 2007; Oostra et al., 2008; Baliji et al., 2009). Recently, Angelini et al. showed that co-expression of full-length nsp3, nsp4 and nsp6 resulted in the formation of CMs and DMVs that resemble to some extent the replicative structures as have been observed in infected cells (Angelini et al., 2013). We previously demonstrated that the membrane-spanning nsps are involved in homo- and heterotypic interactions using co-immunoprecipitation (coIP) and protein complementation assays (Hagemeijer et al., 2011). Furthermore we showed that co-expression of nsp4 with the C-terminal one-third part of nsp3 ($nsp3_C$), which contains the transmembrane domains, redistributes both proteins from the ER into discrete foci that predominantly localize in the perinuclear region of the cell (Hagemeijer et al., 2011). Together these results indicate an important role for the integral membrane nsps in the induction of the CoV replicative structures. In agreement herewith, Sparks et al. have demonstrated that nsp4 is essential for CoV replication (Sparks et al., 2007) and that disruption of the nsp4 glycosylation sites present in the large luminal loop between the first and second transmembrane domain gives rise to aberrant DMVs with detached inner and outer membranes and to a concomitant increased number of CMs (Gadlage et al., 2010).

In view of the importance of the membrane-spanning nsps in CoV replication and the formation of organelle-like replicative structures, we decided to characterize and study the role of these proteins in the induction of membrane rearrangements in more detail, thereby focusing on the membrane rearrangements induced by the co-expression of $nsp3_C$ and nsp4 (see Fig. 1A for a representation of the membrane structure of these proteins). Our results indicate that the foci induced by co-expression of $nsp3_C$ and nsp4 correspond with ER-derived membranes that display increased membrane curvature and resemble the recently described areas of maze-like bodies (MLBs) (Angelini et al., 2013). Importantly, these membrane rearrangements were only observed when the co-expressed $nsp3_C$ and nsp4 proteins were derived from the same CoV species. The specific interplay between the two proteins was attributed to the large luminal loops of nsp3 and nsp4. In agreement herewith, mutation of the conserved cysteine residues in the loop of nsp4 completely abrogated the formation of membrane rearrangements. Finally, we show that the foci induced by co-expression of $nsp3_C$ and nsp4 were able to recruit nsp2 and nsp6 in agreement with the idea that the integral membrane proteins not only function in the biogenesis of the membranous rearrangements, but also in the anchoring of other nsps, onto what will eventually become the replicative structures.

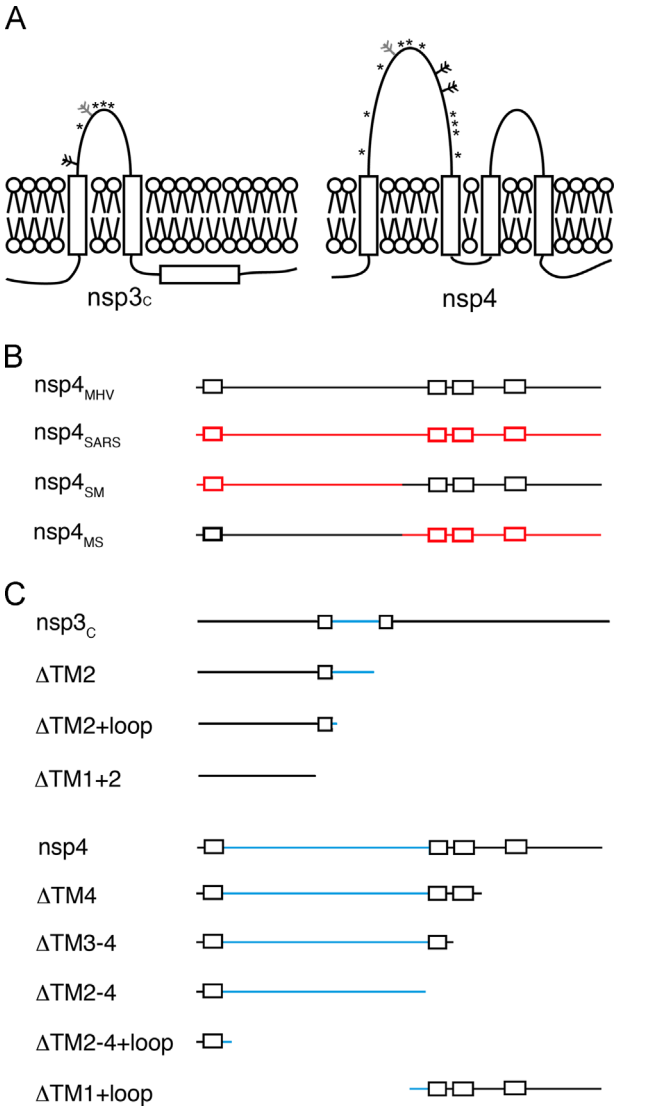

Fig. 1. Overview of CoV $nsp3_C$ and nsp4 constructs used in this study. (A) A schematic representation of the membrane topology of the C-terminal part of nsp3 ($nsp3_C$), and of nsp4. The approximate location of the cysteine residues (*) and *N*-glycosylation sites (-«) in the large luminal loops are indicated, the latter either in gray (SARS-CoV) or in black (MHV-A59). (B) Schematic representation of nsp4 hybrid proteins. Nsp4 sequences derived from MHV or SARS-CoV are indicated by black (MHV) or red (SARS-CoV) lines or rectangles. Rectangles indicate the approximate location of transmembrane domains. $Nsp4_{SM}$ contains the amino-terminal half of SARS-CoV, while its carboxy terminus is derived from MHV. $Nsp4_{MS}$ refers to the reciprocal hybrid protein. (C) Schematic representation of the MHV nsp3 and nsp4 deletion mutants used in this study. Rectangles indicate transmembrane domains and the blue lines indicate either the luminal loop of nsp3 or the large luminal loop of nsp4.

Results

Co-expression of $nsp3_C$ and nsp4 induces ER membrane rearrangements

We have previously shown that $nsp3_C$ and nsp4, which localize to the ER when expressed individually, interact with each other and that their co-expression results in the relocalization of these proteins from the ER to discrete foci mostly concentrating in the perinuclear region of the cell (Hagemeijer et al., 2011). As it is unknown what these punctate structures are, we decided to characterize them in more detail. We first investigated whether the $nsp3_C$ and nsp4 puncta co-localize with specific organelle protein markers such as PDI (ER), GM130 (*cis*-Golgi), EDEM1 (ER