Rotavirus NSP6 localizes to mitochondria via a predicted N-terminal \(\alpha\)-helix

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Specific roles have been ascribed to each of the 12 known rotavirus proteins apart from the non-structural protein 6 (NSP6). However, NSP6 may be present at sites of viral replication within the cytoplasm. Here we report that NSP6 from diverse species of rotavirus A localizes to mitochondria via conserved sequences in a predicted N-terminal \(\alpha\)-helix. This suggests that NSP6 may affect mitochondrial functions during rotavirus infection.

Members of the genus *Rotavirus*, dsRNA viruses of the family *Reoviridae*, cause a large proportion of severe dehydrating gastroenteritis. Rotavirus non-structural protein (NSP) 6 is a protein of \(\leq 98\) aa with unknown function. It is expressed from a \(+1\) alternative ORF in gene segment 11 of rotavirus A and its coding sequence lies entirely within that of NSP5 (Matton et al., 1991). NSP6 has been proposed to localize to sites of rotavirus replication (viroplasms) within the cytoplasm and to interact with NSP5 and NSP2, which are required for viroplasm formation (Matton et al., 1991; Rainsford & McCrae, 2007; Torres-Vega et al., 2000; Viskovska et al., 2014). NSP6 also binds to ssRNA and dsRNA independently of nucleotide sequence (Rainsford & McCrae, 2007). Several rotaviruses encoding defective or truncated NSP6 have been isolated (Ahmed et al., 2005; Kojima et al., 1996; Wu et al., 1998), demonstrating that NSP6 is not absolutely required for replication. Here we showed that NSP6 contains highly conserved N-terminal sequences that direct it to mitochondria.

The gene encoding NSP6 of rhesus rotavirus (RRV; GenBank accession no. EU636934) was cloned by standard methods (Holloway et al., 2009) into pEGFP-N1, pEGFP-C2 (Clontech), pCMV 3-Tag-6 and 3-Tag-8 vectors (Agilent), yielding plasmids encoding NSP6 fused to N-terminal or C-terminal GFP and FLAG tags. Clone integrity was confirmed by sequencing (Holloway et al., 2009). Transfection of MA104 cells grown on glass cover slips in 24-well trays was performed using 1 \(\mu\)g plasmid DNA and either 4 \(\mu\)l Transit LT-1 (Mirus) or 3 \(\mu\)l Fugene HD (Promega) per well, as specified by the manufacturers. Cellular staining with antibody for the FLAG epitope and confocal microscopy were conducted as described elsewhere (Holloway et al., 2014). RRV GFP–NSP6 was found in the cytoplasm and also formed bright cytoplasmic puncta, possibly indicative of aggresomes (Fig. 1a), as described previously (Mohan et al., 2006). RRV FLAG–NSP6 showed mostly cytoplasmic but probably also some nuclear localization (Fig. 1b). RRV NSP6–GFP showing bright fluorescence (30% of transfected cells) was cytoplasmic and nuclear and also formed cytoplasmic puncta (Fig. 1c). The remainder of transfected cells showed very faint but distinct perinuclear and cytoplasmic NSP6–GFP staining that clearly co-localized with mitochondrial protein COXIV (Fig. 1d), as detected using a rabbit mAb to COXIV (Cell Signalling) and Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibodies (Life Technologies). RRV NSP6 expressed with C-terminal FLAG tags showed uniformly moderate fluorescence and also clearly co-localized with COXIV (Fig. 1e). These data showed that RRV NSP6 with C-terminal tags, but not N-terminal tags, localized strongly to mitochondria.

The failure of NSP6 with N-terminal tags to localize to mitochondria suggested the presence of targeting information within the N terminus of the protein. To test this possibility, plasmids expressing RRV NSP6–FLAG with deletions of the first 10 or 20 N-terminal amino acids were constructed. Whereas wt RRV NSP6–FLAG showed complete mitochondrial localization in 90% of cells, NSP6 lacking the first 10 aa was completely cytoplasmic in most (56%) of the transfected MA104 cells, with partial mitochondrial localization in remaining cells (Fig. 1e, f). NSP6 lacking the first 20 aa was completely cytoplasmic in 86% of cells, with the remaining cells showing mostly cytoplasmic localization (Fig. 1e, f). Thus, the first 20
**Fig. 1.** Localization of NSP6 with N- or C-terminal tags in transfected cells. (a–c) MA104 cells were transfected with plasmids expressing RRV NSP6 with N-terminal GFP (GFP–NSP6) (a), N-terminal FLAG tags (FLAG–NSP6) (b) and C-terminal GFP (NSP6–GFP, bright green fluorescing) (c). Fixed cells were stained with antibody to the FLAG epitope (FLAG–NSP6 only) and imaged by confocal microscopy. (d) Cells expressing RRV NSP6–GFP (green) were fixed and stained with antibodies to COXIV (red) and faintly green fluorescing cells were selected for confocal microscopy. The green and red fluorescence intensity profiles in a cross-section (open bar) of merged images are shown (created using ImageJ software, v.1.47, http://imagej.nih.gov/ij/). (e) Representative confocal images and intensity profiles of MA104 cells transfected with plasmids expressing RRV NSP6–FLAG or RRV NSP6–FLAG with deletions of the first 10 aa (NSP6Δ10–FLAG, mostly cytoplasmic) and the first 20 aa (NSP6Δ20–FLAG, mostly mitochondrial).
or 20 aa (NSP6/20–FLAG, completely cytoplasmic) and stained with antibodies to FLAG and COXIV. (f) The localization of RRV NSP6–FLAG, NSP6/10–FLAG and NSP6/20–FLAG was analysed by microscopy of 100 cells in two independent experiments. Mostly mitochondrial denotes that most of the fluorescent signal was observed to be mitochondrial, with some in the cytoplasm. Mostly cytoplasmic denotes that some mitochondrial fluorescence was evident, but most was observed in the cytoplasm. (g) Cells expressing GFP, aa 1–20 of RRV NSP6 fused to GFP (N20–GFP) or aa 1–50 of RRV NSP6 fused to GFP (N50–GFP) were analysed by confocal microscopy for GFP fluorescence. (h) Cells transfected for 24 h to express RRV NSP6–FLAG were infected for 7 h with RRV at an m.o.i. of 0.5, fixed and stained with antibodies to FLAG, COXIV and NSP5, and analysed by confocal microscopy. Images were obtained at ×1000 magnification.

N-terminal amino acids of RRV NSP6 were required for mitochondrial targeting. To test if these residues alone were sufficient to direct mitochondrial targeting, a plasmid encoding the first 20 N-terminal amino acids of RRV NSP6 fused to GFP was constructed (N20–GFP). The localization of N20–GFP was comparable to that of GFP alone, with dispersed nuclear and cytoplasmic fluorescence (Fig. 1g). However, expression from a construct encoding the first 50 N-terminal amino acids of RRV NSP6 fused to GFP (N50–GFP) revealed distinct mitochondrial fluorescence in 58% of 100 randomly selected transfected cells (Fig. 1g). This indicated that in addition to aa 1–20, further information contained between aa 21 and 50 of NSP6 was required for mitochondrial targeting.

As NSP6 has been reported to interact with NSP5 and localize to viroplasms (Matton et al., 1991; Rainsford & McCrae, 2007), the localization of RRV NSP6–FLAG following infection with purified RRV for 7 h at an m.o.i. of 0.5 was assessed, using previously described methods (Holloway et al., 2009). In addition to FLAG and COXIV detection, cells were stained with guinea pig antibodies to rotavirus NSP5, provided by Malcolm McCrae, Pirbright Institute and University of Warwick, UK (Rainsford & McCrae, 2007), and Alexa Fluor 647-conjugated goat anti-guinea pig IgG secondary antibodies (Life Technologies). The NSP6–FLAG remained completely mitochondrial in infected cells showing NSP5 expression (Fig. 1h). NSP5 was largely present in viroplasms dispersed throughout the cytoplasm. Although this absence of NSP6–FLAG from viroplasms does not confirm previous reports (Matton et al., 1991; Rainsford & McCrae, 2007), it remains possible that virally expressed NSP6 localizes to viroplasms. While it appears that NSP6–FLAG already at mitochondria is not translocated to viroplasms during infection, de novo NSP6–FLAG expression following infection may be limited due to translational inhibition and the reported high rate of NSP6 turnover (Rainsford & McCrae, 2007).

In silico analysis of the secondary structure of the N terminus of RRV NSP6 using Jpred 3 (http://www.compbio.dundee.ac.uk/jpred; Cole et al., 2008) predicts a non-amphipathic α-helix (Fig. 2a). The NSP6 of human rotavirus strain Wa (GenBank accession no. JX406757), which shows 4 aa differences from RRV in the first 20 aa, is also predicted to form an α-helix in this region (Fig. 2a). We cloned and sequenced the NSP5/6 gene from avian rotavirus strain Ty-1 (GenBank accession no. KR080697) (Kool et al., 1992). NSP6 of Ty-1 is most similar to that of pigeon rotavirus PO-13 (96% nucleotide identity). Ty-1 NSP6 is highly divergent from RRV NSP6 (41% amino acid identity), although sharing 70% identity in the first 20 aa. Ty-1 NSP6 is also predicted to form an α-helix (Fig. 2a). NSP6 of Wa and Ty-1 strongly localized to mitochondria when expressed with C-terminal FLAGs, showing conservation of the mitochondrial targeting information (Fig. 2b). As avian and mammalian rotavirus A strains are highly divergent (Trojnar et al., 2009), mitochondrial targeting of Ty-1 NSP6 suggests a vital role for this property.

Sequences directing proteins to mitochondria, often referred to as mitochondrial targeting sequences (MTSs), vary widely in composition, length and position within the protein. The best studied MTSs are N-terminal pre-sequences that form amphipathic α-helices, often contain Arg, Leu, Ser and Ala residues and are commonly cleaved after mitochondrial import (Neupert & Herrmann, 2007). Such pre-sequences generally mediate protein import into the matrix or inner membrane. Although the NSP6 N terminus contains several conserved Leu and Arg residues, the non-amphipathic nature of the α-helix and the lack of a predictable cleavage site are suggestive of a cryptic MTS.

To map the MTS, conserved Arg (R) and Leu (L) residues in the N terminus of RRV NSP6 were mutated to Ala (QuikChange II kit; Agilent) and mitochondrial localization assessed by microscopy. Mutated residues comprised the Leu at position 4 (L4), R7, L14 and L15 that are almost completely conserved among all rotavirus A strains and L9 and L11 that are conserved among mammalian but not avian rotavirus A isolates. Substitution of Arg at position 7 with Ala (R7A) strongly affected localization, with NSP6 being mostly or completely found in the cytoplasm in 75% of cells (Fig. 2c, d). The greatest disruption to mitochondrial localization was caused by the L14A substitution, which led to NSP6 being completely (67%) or mostly (32%) cytoplasmic in the vast majority of cells. The single L4A, L9A, L11A and L15A substitutions moderately disrupted localization, with NSP6 completely or mostly localized to mitochondria in 69–87% of cells (Fig. 2d). Very few cells expressing these mutants (0–1%) showed fully cytoplasmic localization of NSP6. Introduction of R7A and L14A together (R7A/L14A) produced complete cytoplasmic localization of NSP6 in 82% of transfected...
Fig. 2. N-terminal sequences required for NSP6 mitochondrial localization. (a) Sequence alignment and secondary structural prediction of the N termini of RRV, Wa and Ty-1 NSP6. Pink cylinders indicate predicted \(\alpha\)-helices. Yellow highlights indicate conserved residues chosen for mutagenesis and localization analysis. (b) MA104 cells were transfected with plasmids expressing Wa and Ty-1 NSP6–FLAG. Fixed cells were stained with antibodies to FLAG and COXIV and imaged by confocal microscopy. Fluorescence intensity profiles of a cross-section (open bar) of merged images are shown. (c) Representative confocal images and intensity profiles of MA104 cells transfected with plasmids expressing RRV NSP6–FLAG with the R7A (mostly cytoplasmic), L14A (mostly cytoplasmic) or R7A/L14A (completely cytoplasmic) substitutions. (d) The localization of RRV NSP6–FLAG with substitution mutations (L4A, R7A, L9A, L11A, L14A, L15A and R7A/L14A) was analysed by microscopy of 100 cells in two independent experiments. Images were obtained at \( \times 1000 \) magnification.
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cells, demonstrating the importance of these two residues within the MTS of NSP6 for mitochondrial targeting (Fig. 2c, d).

Mitochondrial sublocalization was analysed by testing the sensitivity to externally added protease of NSP6 in intact mitochondria and mitoplasts (mitochondria with osmotically ruptured outer membranes) that were produced as before (Baker et al., 2014; Stojanovski et al., 2007). When expressed in transfected HEK 293T cells through the use of 30 µl Fugene HD and 10 µg DNA per 80 cm² flask, RRV NSP6–FLAG showed protease sensitivity in untreated mitochondria, indicating an outer-membrane localization (Fig. 3a, lanes 1 and 2). In contrast, cellular proteins internal to the mitochondria, as detected by Western blotting with antibodies to OPA1 (BD Biosciences), COXIV (Cell Signalling) and SDHA (Abcam) remained protected. Following sodium carbonate extraction (Stojanovski et al., 2007) at pH 10.5 or 11.5, NSP6 and control proteins (detected with anti-cytochrome c antibody; BD Biosciences) were located entirely in the soluble fraction (Fig. 3b). The integral membrane proteins Sam50 (Humphries et al., 2005) and mitofusin 2 were found in the insoluble fraction (pellet). These data suggest that NSP6 is peripherally associated with the outer mitochondrial membrane (Fig. 3b). Consistent with this, in silico analysis of NSP6 does not predict a hydrophobic region suggestive of a transmembrane region (data not shown) like that found in mitochondrial outer-membrane proteins MAVS and Bcl-2 (Nguyen et al., 1993; Seth et al., 2005). The NSP6 outer-membrane localization and the absence of NSP6 cleavage products, as shown by the presence of the full-length 15 kDa protein alone in Western blots (data not shown), are not consistent with classical pre-sequence MTS targeting. These data provide further evidence that the NSP6 N terminus contains a cryptic MTS.

Many viral proteins localize to mitochondria and modulate mitochondria-related functions to benefit the virus (Castanier & Arnoult, 2011). Such virus-targeted processes include apoptotic cell death, triggered by the release of factors like cytochrome c from the mitochondrial intermembrane space (Tait & Green, 2013). As rotavirus induces apoptosis in certain cell lines and enterocytes in vivo, NSP6 possibly could modulate apoptosis (Halasz et al., 2010; Martin-Latil et al., 2007; Superti et al., 1996). NSP6 also might interfere with mitochondrial MAVS, a crucial player in innate immune signalling and type I IFN induction by rotavirus (Broquet et al., 2011; Holloway & Coulson, 2013; Sen et al., 2011). Although we found no evidence of an interaction between ectopically expressed NSP6 and viroplasms, previous studies showing an NSP6 interaction with NSP5 and NSP6 presence in viroplasms during infection (Matton et al., 1991; Torres-Vega et al., 2000) suggest that NSP6 might link viroplasms to mitochondria, perhaps to provide energy for virus replication. Several other viruses, including reovirus, replicate at cytoplasmic sites that appear to associate with mitochondria

![Fig. 3. Submitochondrial localization of NSP6.](http://jgv.microbiologyresearch.org)

(a) NSP6–FLAG
Mitoplasts - - + +
Mitochondria + + - -
PK - - + +

(b) NSP6–FLAG
\[\text{Na}_2\text{CO}_3\]
\begin{array}{c}
\text{pH 10.5} \\
\text{P}
\end{array}
\begin{array}{c}
\text{pH 11.5} \\
\text{S}
\end{array}

NSP6–FLAG (15 kDa)
Sam50 (52 kDa)
Mitofusin 2 (80 kDa)
SDHA (70 kDa)

Plastic

Integrals

Peripheral

Cytochrome c (12 kDa)
(Fernández de Castro et al., 2014). Further study of these possibilities is warranted, which should shed more light on the elusive role of NSP6 in rotavirus biology.

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**References**


