Nucleolar-cytoplasmic shuttling of PRRSV nucleocapsid protein: a simple case of molecular mimicry or the complex regulation by nuclear import, nucleolar localization and nuclear export signal sequences

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Abstract

The order Nidovirales, which includes the arteriviruses and coronaviruses, incorporate a cytoplasmic replication scheme; however, the nucleocapsid (N) protein of several members of this group localizes to the nucleolus suggesting that viral proteins influence nuclear processes during replication. The relatively small, 123 amino acid, N protein of porcine reproductive and respiratory syndrome virus (PRRSV), an arterivirus, presents an ideal model system for investigating the properties and mechanism of N protein nucleolar localization. The PRRSV N protein is found in both cytoplasmic and nucleolar compartments during infection and after transfection of gene constructs that express N-enhanced green fluorescent protein (EGFP) fusion proteins. Experiments using oligopeptides, truncated polypeptides and amino acid-substituted proteins have identified several domains within PRRSV N protein that participate in nucleo-cytoplasmic shuttling, including a cryptic nuclear localization signal (NLS) called NLS-1, a functional NLS (NLS-2), a nucleolar localization sequence (NoLS), as well as a possible nuclear export signal (NES). The purpose of this paper is to review our current understanding of PRRSV N protein shuttling and propose a shuttling scheme regulated by RNA binding and post-translational modification.
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1. Introduction

The localization of cytoplasmic RNA virus nucleocapsid (N) protein antigens to the nucleus/nucleolus implicates a potential role for the N protein in the regulation of nuclear processes during virus replication. However, to date only N proteins from a few genera have been studied to the extent of identifying potential localization domains and interactions with nuclear/nucleolar processes or proteins. The N protein of Semliki Forest virus (SFV), a flavivirus, contains two nucleolar localization sequences (NoLS; Favre et al., 1994). Localization is implicated in the modulation of translation by either facilitating or blocking ribosome assembly in the nucleolus (Elgizoli et al., 1989). The p21 form of the hepatitis C virus (HCV) N protein is translocated into the nucleus (Yasui et al., 1998) where it participates in the cytopathogenesis of HCV infection, perhaps by blocking nuclear import and export of host cell proteins/mRNAs (Isoyama et al., 2002) or by facilitating cell transformation (Yamanaka et al., 2002).

In 1999, we reported the nucleolar localization of the N protein of porcine respiratory syndrome virus (PRRSV), an arterivirus (Rowland et al., 1999). PRRSV N protein is distributed between nucleolar and cytoplasmic compartments in cultured macrophages and MARC-145 cells infected with North American and European PRRSV genotypes. A similar pattern of cytoplasmic/nucleolar localization has been reported for several members of the coronavirus family, including the group III coronavirus, infectious bronchitis virus (IBV), the group I coronavirus, transmissible gastro-

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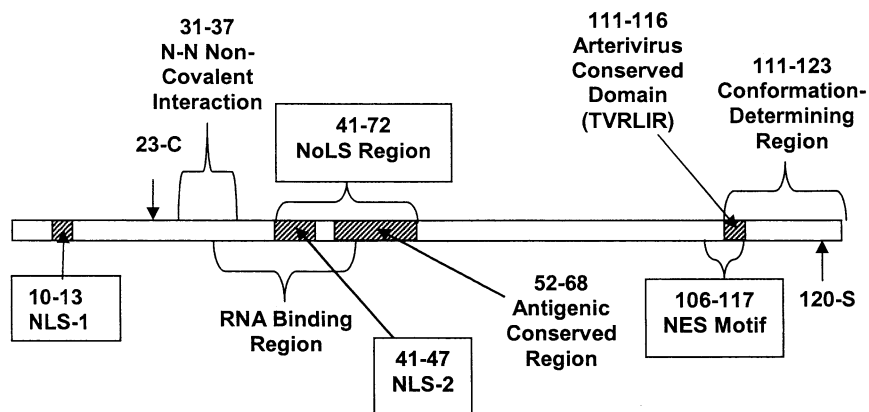*E-mail address:* browland@vet.ksu.edu (R.R.R. Rowland).

Fig. 1. Structural and functional domains within PRRSV N protein. The diagram identifies the location of domains typically found in North American PRRSV isolates. The numbers identify the amino acid positions covered by each domain. 23-C identifies the location of the conserved cysteine residue involved in the formation of covalent bonds between N protein molecules. 120-S identifies the location of a serine residue that is a target of phosphorylation. The regions in boxes are the putative localization domains. For details see text.

enteritis virus (TGEV), and the group II coronavirus, mouse hepatitis virus (MHV; Hiscox et al., 2001; Wurm et al., 2001). More recently, Tijms et al. (2002) reported the nucleolar localization of the N protein from equine arteritis virus (EAV), another member of the arterivirus group. PRRSV, coronavirus and EAV N proteins when expressed alone or fused to the red-shifted enhanced green fluorescent protein (EGFP) localize to the nucleolus demonstrating that translocation across the nuclear pore complex (NPC) and accumulation in the nucleolus are independent of other viral proteins (Rowland et al., 1999; Hiscox et al., 2001; Wurm et al., 2001).

Similar to SFV, the N proteins of the arteriviruses may participate in the modulation of nuclear processes during virus replication. Another possibility is that nuclear/nucleolar localization is an example of molecular mimicry, i.e. RNA binding domains which are typically enriched in lysine and arginine residues also mimic nuclear and nucleolar localization signal sequences (LaCasse and Lefebvre, 1995). However, our current data have identified at least four separate domains, which may participate in nuclear translocation, nucleolar localization, nuclear export and cytoplasmic retention indicating that the nucleolar-cytoplasmic shuttling of N is a complex and highly regulated process.

2. PRRSV N protein structure

The arteriviruses are enveloped positive-stranded RNA viruses resembling togaviruses in morphology but use a replication strategy similar to coronaviruses (Lai, 1990; Snijder and Meulenberg, 1998). Based on the use of a similar replication strategy, the toroviruses, coronaviruses and arteriviruses belong to a recently created order, Nidovirales (Cavanagh, 1997). Other members of the arterivirus group are lactate dehydro-genase-elevating virus (LDV) of mice, EAV and simian hemorrhagic fever virus (SHFV; see Plagemann, 1996 for review). Based on genetic and antigenic information, PRRSV isolates can be further divided into two distinct genotypes (Nelsen et al., 1999). The North American genotype is represented by isolates similar to VR-2332, described by Benfield et al. (1992), whereas the European genotype is represented by isolates genetically similar to the Lelystad virus (Wensvoort et al., 1991).

The 15 kDa non-glycosylated PRRSV N protein, translated from ORF 7, forms the principal component of N. The N proteins of VR-2332 and Lelystad viruses do not possess a cleavable signal peptide and are maintained as mature 123 and 128 amino acid proteins, respectively (Mardassi et al., 1996). The N protein is the most abundant viral protein in virus-infected cells and constitutes as much as 40% of the protein content of the virion (Snijder and Meulenberg, 1998). A summary of the structural and functional domains within the N protein is illustrated in Fig. 1. Both covalent and non-covalent bonds participate in the formation of homo-dimers, Ns and other higher ordered structures. Covalent interactions are formed through disulfide linkages between conserved cysteines at position 23 in North American strains and position 27 in European isolates. More recently, one of us reported that non-covalent interactions, involving residues 31–37, also contribute to N protein polymerization (Wootton and Yoo, 2003). The reason for both soft (non-covalent) and hard (covalent) docking interactions between N proteins is not clear, but suggests that different types of interactions may be involved in determining how N functions in its structural and non-structural capacities.

One interesting aspect of N protein polymerization is the role of RNA. The domain involved in forming non-covalent interactions between N protein molecules lies between two basic regions, identified as NLS-1 and NLS-2 in Fig. 1. At least one basic region forms part of

a putative RNA binding domain, which likely includes amino acid residues 34 through 51 (Daginakatte and Kapil, 2001). Non-covalent N–N associations are weakened in the presence of RNase or when the two adjacent basic regions are removed (Wootton and Yoo, 2003).

Recently, we reported that the N protein exists as a phosphoprotein in cells during replication and in the intact virion (Wootton et al., 2002). Furthermore, phosphorylation occurs in cells transfected with the N gene, demonstrating that cellular kinases are involved in phosphorylation. Phosphoamino acid analysis combined with site-directed mutagenesis indicates that phosphorylation is restricted to several serine residues. The actual number and location of serine residues targeted by kinases and the level of phosphorylation during virus replication and virion assembly are not known, but at least some N proteins may exist in a hyperphosphorylated state. Recently, we identified the serine at position 120 as one site for phosphorylation. However, the 120-serine is not present in all North American isolates (personal observation).

The N protein is highly immunogenic in pigs and in mice. Accordingly, numerous panels of monoclonal antibodies (mAbs) have been generated for which major epitopes lie predominantly in the central region of the protein (Rodriguez et al., 1997; Wootton et al., 1998) in an area of high surface probability. Even though the North American and European PRRSV N proteins are only about 63% identical at the amino acid level, mAb binding studies show a common antigenic region between amino acids 52 and 69, located within a possible loop domain (Rodriguez et al., 1997; Meulenberg et al., 1998; Wootton et al., 1998). Recognition by several mAbs is lost after single amino acid substitutions within the last 11 amino acids, indicating a change in the overall conformation of the protein. Therefore, we have termed this region the conformation-determining region (Wootton et al., 1998). As discussed later, anti-N mAbs have proved useful for probing conformational changes that result from mutations that produce a change in N protein localization.

3. Identification and functional characterization of N protein nuclear import signal sequences

Classical nuclear localization signal (NLS) sequences incorporate regions enriched in basic amino acids and generally conform to one of three motifs (Nakai and Kanehisa, 1992). The "pat4" NLS consists of a continuous stretch of four basic amino acids (lysine or arginine) or three basic amino acids associated with histidine or proline. The "pat7" NLS starts with a proline and is followed within three residues by an amino acid sequence containing three basic residues out of four. The third type of NLS, known as a "bipartite" motif, consists of two basic amino acids, a 10 amino acid spacer and a five amino acid sequence containing at least three basic residues. This information has been incorporated into computer programs that identify NLS motifs (Nakai and Kanehisa, 1992), but cannot always successfully predict the localization of a protein to the nucleus (Silver, 1991; Hicks and Raikhel, 1995). Proteins without an NLS can be co-transported with other nuclear proteins and proteins that contain NLS sequences may remain cytoplasmic, especially if NLS sequence is blocked or buried within the protein (Roberts et al., 1987). Finally, small proteins less than 50–70 kDa can passively diffuse through NPC (Paine et al., 1975; Gorlich and Kutay, 1999).

The approach for the identification of functional NLS sequences in PRRSV N protein consisted of three steps. The first was the use of peptide sequence analysis programs such as PSORT (Nakai and Kanehisa, 1992) to locate classical NLS motifs. PSORT analysis of the North American PRRSV isolate, SDSU-23983, identified a pat4 domain at position 10 called NLS-1 and a pat7 motif at position 41 called NLS-2 (Figs. 1 and 2). Furthermore, a Hopp–Woods hydrophilicity analysis (Hopp and Woods, 1981) of the peptide sequence, shown in Fig. 2, predicts that both NLSs are in hydrophilic regions and should be easily accessible. However, it is important to note that the antigenic conserved region of the native protein, recognized by most conformation-dependent mAbs, is in close proximity to NLS-2 (Fig. 1).

The second step in the analysis was to determine if each putative NLS could localize a protein tag to the nucleus. For the purpose of these experiments, we chose the autofluorescent EGFP as a tag for N oligopeptides. EGFP by itself, because of its relatively small size (approximately 25 kDa) and neutral properties, maintains a localization pattern consistent with passive diffusion. Therefore, EGFP is found in both the cytoplasm and nucleoplasm, but is excluded from the nucleolus and some cytoplasmic compartments (see Fig. 3a; Rowland et al., 1999). The diffusion of EGFP throughout the cell offers a special advantage, by allowing the study of the localization properties of import or export signal sequences or their combined function. When EGFP is fused to the N protein, as shown in Fig. 3b, the distribution of EGFP tag changes, with a large accumulation of N in the nucleolus and disappearance from the nucleoplasm. Cytoplasmic localization is largely unaffected except for an increased accumulation of N-EGFP in the perinuclear region. N protein gene fragments containing NLS-1 (amino acids 1–14) or NLS-2 (amino acids 39–48) were ligated in front of EGFP and the fusion gene placed under control of a CMV promoter. Both peptides were able to accumulate EGFP in the nucleus as well as the
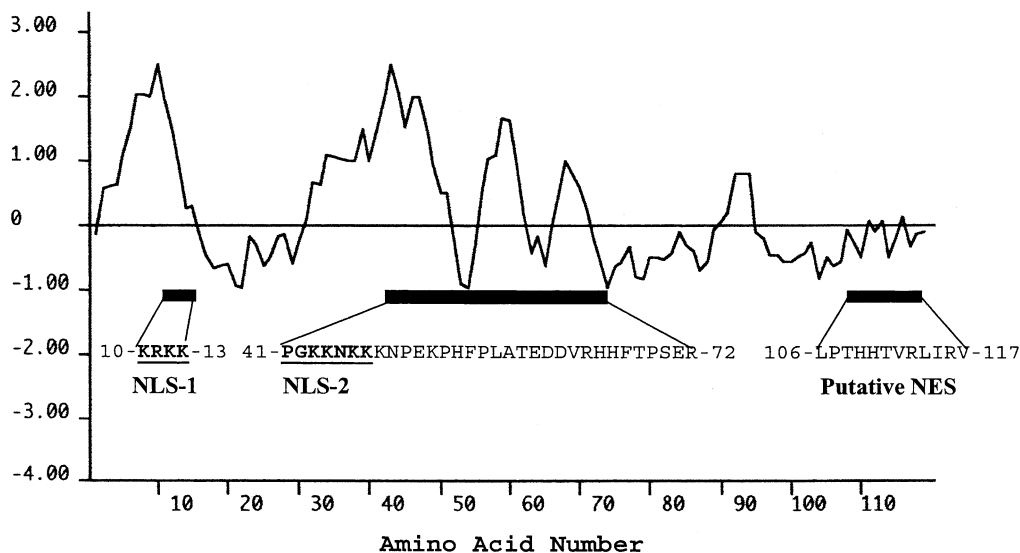
Fig. 2. Hydrophilicity plot of PRRSV N protein. Basic regions that form potential NLS sequences are identified as NLS-1 and NLS-2. A leucine-rich region between 106 and 117 forms a potential NES with an LXL-like motif. The hydrophilicity plot is based on the analysis of peptide sequence according to Hopp and Woods (1981).

nucleolus, indicating that NLS-1 and NLS-2 can function as NLS sequences, sufficient to localize EGFP to the nucleus (Fig. 3c and d). However, these data provided no information on which NLS or if both participate in N protein translocation to the nucleus. Therefore, the third step in the analysis was to study the localization of N proteins that contained mutations in each or both of the localization domains. In summary (see Rowland et al., in press), the substitution of lysines for glycines at positions 10 and 11, which changed the pat4 NLS-1 sequence from 10-KRKK to 10-GGKK in the construct EGFP-N (1–123, G10, G11), did not alter N-EGFP localization. Even the complete elimination of NLS-1 by removing the first 14 residues from the N-terminus (construct EGFP-N (14–123)) produced a localization pattern similar to wild-type N. To eliminate NLS-2, we substituted lysines 43 and 44 with glycine residues, changing the NLS-2 sequence from 41-PGKKNKK to 41-PGGGNKK. The resulting construct, EGFP-N (1–123, G43, G44), remained in the cytoplasm and was completely excluded from the nucleus/nucleolus. These results suggested that lysines 43 and 44 formed part of a functional NLS sufficient for the translocation of N into the nucleus. However, we recognized that the elimination of lysines in NLS-2 could produce a conformational change in the N protein, which might cause refolding and loss of NLS accessibility. We took advantage of an array of six anti-N mAbs to identify possible changes in N protein conformation. The mAbs used were SDOW-17, SR-30, VO-17, JP-24, EP-47 and MR-40 (Nelson et al., 1993; Wootton et al., 1998, 2001). All antibodies reacted with the full-length N-EGFP fusion protein indicating that EGFP did affect N protein conformation. Furthermore,

the same panel reacted with both NLS-1- and NLS-2-minus mutant proteins. From these data, we concluded that NLS-2 is the signal sequence involved in the transport of N into the nucleus.

This data indicate that NLS-1 is a cryptic localization signal, which alone can function as an NLS, but is inactive in the context of the native protein. However, nuclear localization activity from NLS-1 is evident when the conformation of the N protein is altered. For example, removal of eight amino acids from the C-terminal end within the conformation-determining region (Fig. 1) is sufficient to eliminate reactivity with anti-N mAbs and to restore nucleolar localization to the NL-2-minus construct. The further removal of NLS-1 resulted in the loss of nuclear/nucleolar localization (Rowland et al., in press). The role that NLS-1 plays in nuclear translocation of N during virus replication remains to be determined. It is possible that small conformational changes caused by RNA binding or phosphorylation, discussed later, may regulate NLS-1 activity.

4. Peptide region covered by amino acids 41–72 contains the nucleolar localization signal sequence

Proteins that localize to the nucleolus typically possess an NoLS motif. The NoLS associated with most viral proteins is usually no longer than 20 amino acids residues and possesses at least nine basic residues, including one continuous stretch of four basic amino acids or two stretches of three basic amino acids. An NLS is usually embedded within the NoLS peptide sequence (Kubota et al., 1999). The analysis of PRRSV
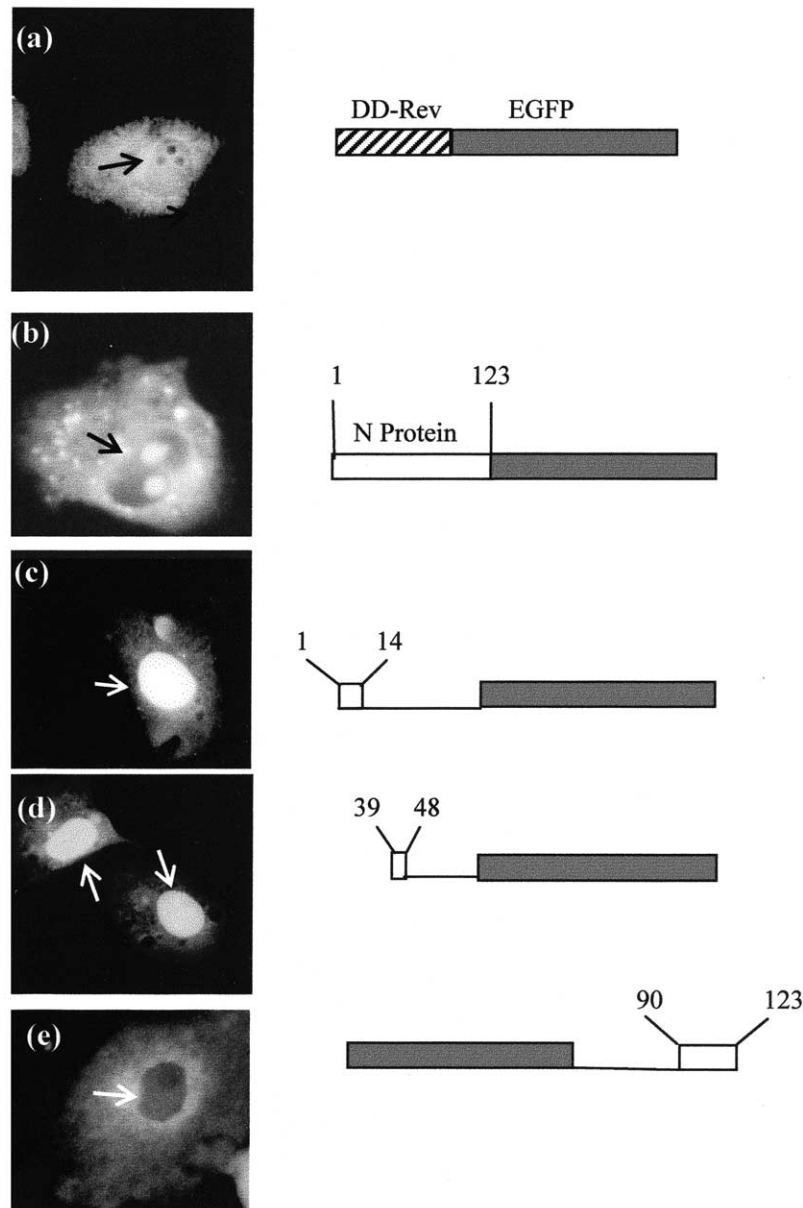
Fig. 3. Intracellular localization of N oligopeptide-EGFP fusion proteins. MARC-145 cells were transfected with the constructs illustrated on the right. The numbers identify the location of the oligopeptide region in the N protein. Cells were transferred to 10-well slides and allowed to adhere overnight. Slides were fixed in 100% acetone and photomicrographs taken at high power on a fluorescence microscope. An NLS-minus, NES-minus ERev protein, called DDRev represented by a hatched bars, was used as an irrelevant peptide to stabilize EGFP during acetone fixation. Arrows identify the location of the nucleus in each cell. The dark spots within the nucleus of (a) and bright spots in the nucleus of (b) are the nucleoli.

N protein surrounding NLS-1 and NLS-2 shows no similarities to other viral NoLS motifs. Therefore, to determine where the NoLS of N was located we performed truncations of the N protein from the N- and C-terminal ends. In summary, EGFP fusion proteins containing fragments 41–123 and 1–72 localized to the nucleolus, whereas peptides 42–123 and 1–71 showed a marked increase in the accumulation of the EGFP tag in the nucleoplasm (Fig. 4; Rowland et al., in press). EGFP construct that contained the 41–72 peptide produced a localization pattern similar to N-

EGFP. This location for NoLS overlaps with the predicted NoLS proposed by Hiscox et al. (2001). However, both NoLS sequences incorporate NLS-2. A comparison with known viral NoLS sequences shows little sequence homology between N and other viral NoLS sequences, except for the incorporation of an NLS (see Fig. 5). What make PRRSV N protein NoLS unique are its relatively long length, an overall paucity of basic residues and the inclusion of a stretch of acidic amino acid residues, EDD, beginning at position 60. Current work is directed at further resolving PRRSV
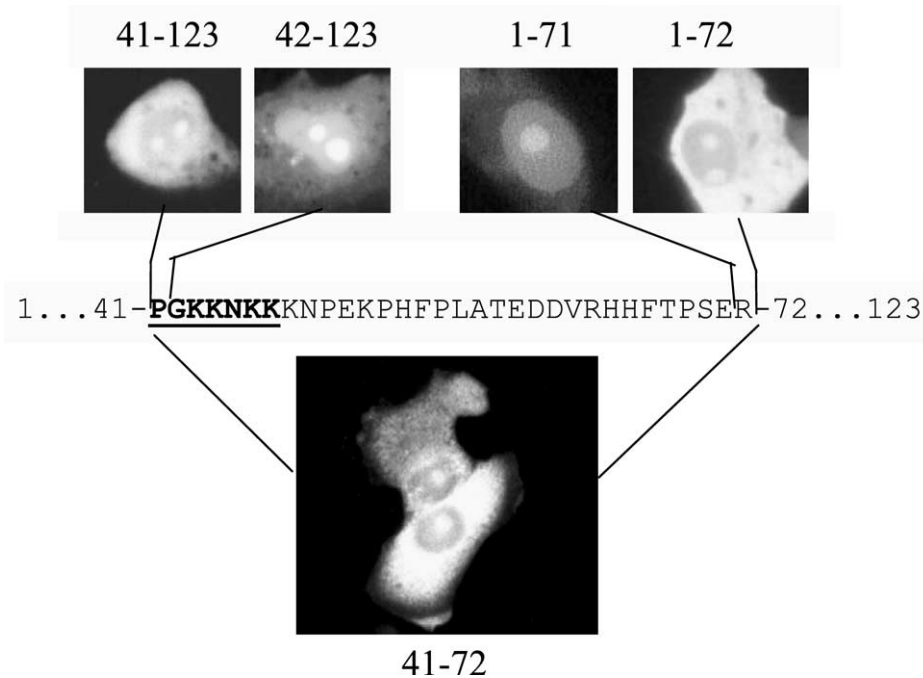
Fig. 4. Identification of the region in PRRSV N protein containing a possible NoLS. Photomicrographs showing the localization patterns of peptide-EGFP fusion proteins. The numbers above or below each photomicrograph identify the region of the N protein used to make the fusion protein. Constructs were expressed in MARC-145 cells.

NoLS sequence, including the contribution of individual amino acid residues.

5. The association of N with cellular proteins involved in nuclear transport

NLS-dependent translocation of most cellular and viral proteins across NPC is initiated by the binding of an NLS on the cargo protein to importin-α in the cytoplasm. The cargo/importin-α complex binds to β-importin and is then targeted to NPC. In the presence of additional accessory proteins, including Ran-GDP, the cargo/importin complex is transported across NPC and released into the nucleoplasm (Gorlich and Kutay, 1999). Modifications to this pathway include the substitution of importin-β for the importin-α/β heterodimer

in the initial recognition of the cargo protein (Lam et al., 2001; Henderson and Percipalle, 1997).

The approach we used to study N protein–importin interactions was a modified pull-down assay in which importin proteins are fused to GST and incubated with labeled N proteins. The importin-N complex is removed using glutathione-Sepharose (Rowland et al., in press). Labeled N proteins, obtained from either PRRSV-infected cells or from in vitro translation products, were able to bind both GST-importin-α and GST-importin-β, which indicate that PRRSV N is capable of interacting with importin receptor proteins and provides further evidence for NLS-dependent transport of N through NPC.

Even though these data suggest an importin-mediated import pathway, we have not ruled out the possibility for other entry mechanisms. For instance, nucleolar

Viral Protein	Sequence	Reference
HIV-1 Rev	RQARRN**RRRR**WRERQR	(Kubota et al., 1989)
HIV-1 Tat	GR**KKRR**QRRRAHQ	(Dang and Lee, 1989)
HTLV-1 Rex	**PKTRRRPRR**SQRKRPPTP	(Siomi et al., 1988)
Semliki NoLS-1	KP**KKKK**TTKPKPKTQP	(Favre et al., 1994)
Semliki NoLS-2	**RRRKR**NRDAA**RRRRK**Q	(Favre et al., 1994)
MDV MEQ	**RRRKR**NRDAA**RRRRK**	(Liu et al., 1997)
PRRSV N	**PGKKNKK**KNPEKPHFPLATEDDVRHHFTPSER	

Fig. 5. Comparison of the potential PRRSV N protein NoLS with known viral NoLS sequences. Bold letters identify classical NLS motifs (Nakai and Kanehisa, 1992). Underlined letters are basic amino acids.

proteins, such as nucleolin, fribrillarin and B-23, are found distributed between nucleolar and cytoplasmic compartments, a pattern of distribution resembling the N protein. Nucleolin is the most abundant protein in the nucleoli of rapidly dividing cells. The molecular basis for nucleolin's participation in nucleolar biogenesis and other proliferation-related functions can be localized to one of three domains. The N-terminal one-third is enriched in acidic residues, which participate in the reorganization of the rDNA chromatin regions (Srivastava and Pollard, 1999) and can interact with stretches of basic amino acids in NoLS sequences, thus facilitating the transport of proteins from the cytoplasm to the nucleolus (Chen et al., 2002). B-23 is also enriched in acidic amino acid residues. The NoLS in the HTLV-1 Rex protein can interact with B-23, which acts as shuttle protein with the capacity to transport Rex from the cytoplasm to the nucleolus (Adachi et al., 1993). A similar mechanism of nucleolar transport has been described for HIV Tat protein (Liu et al., 1997).

6. The N protein contains a possible nuclear export signal sequence

Protein export from the nucleoplasm to the cytoplasm usually incorporates a nuclear export signal (NES) sequence. NES sequences are relatively short linear oligopeptides, but unlike NLS and NoLS domains they are enriched in leucine residues. The classical NES is composed of the peptide sequence X-$R_{(2-4)}$-X-R_2-X-R-X, where X is leucine, isoleucine or valine and R any amino acid (Hope, 1999; Henderson and Eleftheriou, 2000). Atypical NES sequences are defined as CRM1-dependent, but do not contain the classical NES motif. Examples include EIAV and FIV Rev proteins (Otero et al., 1998). In addition to CRM1-dependent export, CRM1-independent nuclear export has been identified in variety of viral proteins, including human adenovirus E1B-55K, CMV pUL69 and HSV-1 ICP27 (Dosch et al., 2001; Lischka et al., 2001; Soliman and Silverstein, 2000). PRRSV N protein contains two regions, which are sufficiently enriched in hydrophobic amino acids to function as NES motifs. The first hydrophobic region is located between amino acids 19 and 30, but does not contain an LXL motif. The second hydrophobic region, which contains at least one LXL motif (see Fig. 2), is located between amino acids 106 and 117. The same sequence is found in the European Lelystad N protein. A comparison of the putative N protein NES with known viral NES sequences is shown in Fig. 6.

To determine if the N protein NES sequence could export a protein from the nucleus, we placed amino acid residues 90–123 in front of EGFP and followed the localization of the EGFP fusion protein in MARC-145 cells. This construct produced no result, i.e. the localization pattern was similar to EGFP. However, when the 90–123 peptide was switched so that the N peptide fragment was on the C-terminal end of EGFP (the orientation found in the native N protein), localization was predominantly cytoplasmic (Fig. 3e). This result indicates that the C-terminal region of N contains an NES. Current work is directed at further identifying the amino acids involved in NES function.

Classical and atypical NES sequences function by forming an interaction with CRM1, the principal export shuttle protein. We applied the GST-bead pull-down assay, described earlier, to determine if CRM1 could bind N. Radiolabeled N protein, prepared from virus-infected cells or by in vitro translation, was incubated with the GST-human CRM1 fusion protein immobilized on glutathione-Sepharose beads. The results showed that CRM1 indeed bound to PRRSV N protein (Rowland et al., in press).

7. Regulation of N protein shuttling

PRRSV and coronavirus N proteins do not appear in the nucleoli of all cells during infection or after transfection with the N gene fused to EGFP. Hiscox et al. (2001) reported up to 90% of cells that contained coronavirus N only in the cytoplasm. In previous work, we reported as few as 20% of PRRSV-infected MARC-145 cells that contained detectable quantities of N in the nucleoli (Rowland et al., 1999). Furthermore, the percentage of PRRSV-infected cells with N in the nucleolus is further dependent on when observations are made during the course of infection. In contrast to these results, Tijms et al. (2002) reported that EAV N protein localized to all nucleoli when cells were treated with leptomycin B (LMB), an inhibitor of CRM1-dependent export. In our studies, typically 50–70% of transfected cells localize N-EGFP protein to the nucleolus. These observations suggested to us that arterivirus and coronavirus N proteins localize to the nucleolus of all cells; however, the absence of detectable amounts of N in the nucleolus may be the result of regulation, e.g. enhanced nuclear export of N relative to import in some cells.

Tartakoff et al. (2000) described several schemes for the shuttling of proteins between the cytoplasm and the nucleus. Viral proteins, such as HIV Rev, which contains single NLS and NES domains, continuously shuttle back and forth between the cytoplasm and the nucleus, an efficient mechanism for recycling Rev function. This type of nucleo-cytoplasmic shuttling scheme is described as $[C-N-C]_n$ (abbreviated CNC-N), where C and N represent cytoplasmic and nuclear compartments, respectively, and the subscript n indicates an unlimited number of trips between the C and N

Viral Protein	NES Sequence	Reference
HIV Rev	LGLPPLER**LTL**	(Fischer et al., 1995)
HSV ICP27	IDMLIDLG**LDL**	(Sandri-Goldin, 1998)
EIAV Rev	PLESDQWCRVLRQSLPEEKIP	(Fridell et al., 1993)
FIV Rev	KKMMTDLEDRFRKLFGSPSKDEYT	(Mancuso et al., 1994)
PRRSV N	106-LPTHHTVRL**IRV**	

Fig. 6. Comparison of the N protein hydrophobic region with known viral NES sequences. Underlined regions are the hydrophobic amino acid residues and bold letters identify the conserved LXL motif present in classical NES sequences.

compartments (see illustration presented in Fig. 7). In contrast, ribosomal proteins are synthesized in the cytoplasm, transported through NPC into the nucleolus, where they are combined with rRNA and assembled into ribosomal subunits, then transported back to the cytoplasm, making only a single trip through the nucleus/nucleolus. This type of shuttling scheme is described as $[C–N–C]_1$ (the subscript 1 indicates a single trip from the cytoplasm to the nucleus and then back to the cytoplasm, and for the purpose of this review it is abbreviated as CNC-1).

The two types of shuttling can be distinguished based on the ability of LMB and cyclohexamide (CHX) to interrupt localization during the steady-state expression of EGFP-tagged proteins. LMB is a specific inhibitor of nuclear export and functions by covalently binding to the exportin shuttle protein, CRM1, thus preventing the
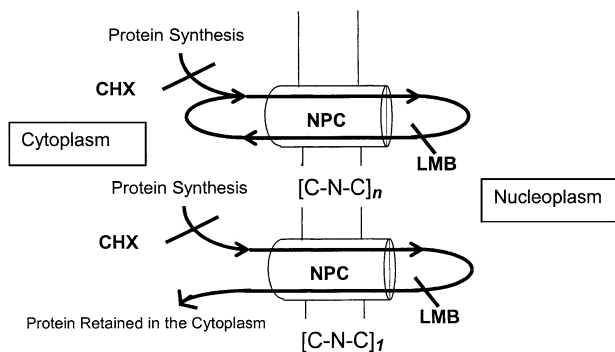

Fig. 7. Shuttling schemes for nucleo-cytoplasmic proteins. The upper figures illustrate the shuttling scheme for CNC-N proteins. An NLS domain localizes the protein to the nucleus. Association of CRM1 with the NES domain then shuttles the protein back to the cytoplasm, where it is re-transported back to the nucleus. The cyclic nature of this trafficking scheme means that the steady-state distribution is largely independent of de novo protein synthesis and not affected by protein synthesis inhibitors such as CHX. However, LMB, an inhibitor of CRM1, blocks export, which leads to the eventual accumulation of protein in the nucleus. The lower figure shows the trafficking scheme typical of CNC-1 proteins which are transported to the nucleus and then returned to the cytoplasm, cycling through the nucleus only once. The steady-state distribution of CNC-1 proteins is dependent on de novo protein synthesis; therefore, in the presence of CHX, protein will disappear from the nucleus. In the presence of LMB, protein export is blocked. LMB does not affect the distribution of protein molecules retained in the cytoplasm.

formation of the cargo protein–CRM1 export complex (Fornerod et al., 1997). In the absence of both drugs, CNC-N and CNC-1 proteins are distributed between both cytoplasmic and nuclear compartments, a product of the rates of nuclear import versus export. In cells that express equine infectious anemia virus Rev protein linked to EGFP (ERev-EGFP) or N-EGFP, this steady-state equilibrium is represented by cells that contain EGFP-labeled protein in both cytoplasmic and nuclear/nucleolar compartments (Fig. 8a and e). However, in any given cell, this distribution may be skewed towards the preferential accumulation of EGFP-labeled protein in the nucleus or the cytoplasm. In the presence of LMB, CNC-N proteins preferentially accumulate in the nucleus, a result of the loss of export function (Fig. 8b). Since the shuttling of CNC-N proteins is not dependent on ongoing protein synthesis (see Fig. 7), CHX does not affect the distribution of ERev-EGFP in the presence or absence of LMB (Fig. 8). In contrast, the addition of CHX to CNC-1 proteins leads to the eventual disappearance of protein from the nucleus, because only newly synthesized proteins are imported into the nucleus, then exported and not allowed to re-enter the nuclear compartment. In the presence of CHX, the amount of N-EGFP in the nucleolus is greatly reduced in all cells (Fig. 8, compare e with g). However, the addition of LMB restores nucleolar localization by preventing the exit of proteins already present in the nucleus (Fig. 8h).

One interesting observation was the effect of LMB on the distribution of N-EGFP. Normally, 50–70% of transfected cells show the cytoplasmic-nucleolar pattern of N-EGFP localization. In the remaining cells, N-EGFP is found only in the cytoplasm. However, after the addition of LMB, all fluorescent cells exhibited the cytoplasmic-nucleolar pattern of localization, indicating that N-EGFP localizes to the nucleolus of all cells, which confirms the observation of Tijms et al. (2002) who found EAV N protein in the nucleoli of all cells after treatment with LMB. Consistent with the CNC-1 model, a certain amount of N-EGFP is retained in the cytoplasm in the presence of LMB (Fig. 8f).

The results from CHX and LMB treatment experiments verify that ERev functions as a CNC-N protein.
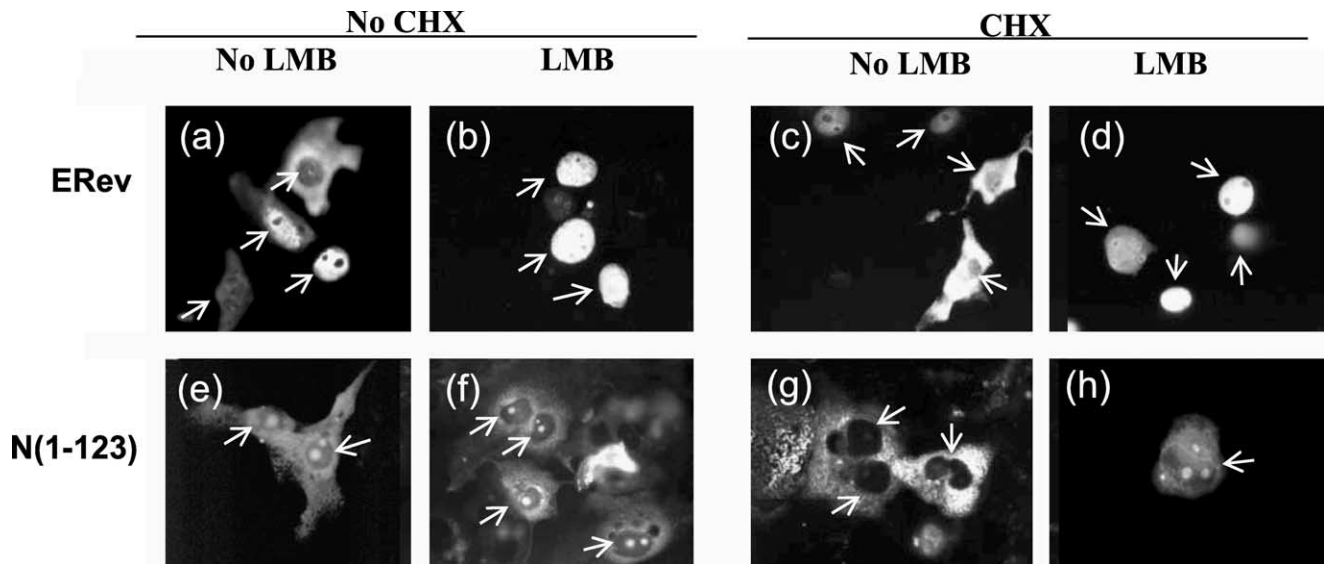
Fig. 8. The effect of LMB and CHX on the localization of ERev-EGFP and N-EGFP proteins. MARC-145 cells were transfected with ERev- or N-EGFP fusion constructs. After 15 h, CHX and LMB were added at concentrations of 50 μg/ml and 100 ng/ml, respectively, and slides incubated for an additional 8 h. Cells were fixed in acetone prior to visualization. Arrows identify the location of the nuclei.

In contrast, the N protein behaves in a manner consistent with a CNC-1 protein. The mechanism for the retention of N in the cytoplasm and the significance of the CNC-1 shuttling scheme in virus replication are not well understood and are discussed in more detail below. However, the limited cycling of N through the nucleus means that N is eventually returned to the cytoplasm where it can participate in viral assembly.

Phosphorylation is one of the principal post-translational modifications involved in the regulation of nucleo-cytoplasmic shuttling of viral and cellular proteins (Jans and Hubner, 1996; Komeili and O'Shea, 1999). The negatively charged phosphate groups, when placed in close proximity to NLS, can neutralize the positively charged lysine and alanine residues, effectively blocking interactions with shuttle proteins. Phosphorylation can also enhance nuclear transport, by inducing conformational changes, that increases the accessibility of NLS (Jans and Hubner, 1996; Xiao et al., 1998). The role that phosphorylation plays in cytoplasmic-nucleolar shuttling of N is unknown. Our most recent data indicate that both phosphorylated and non-phosphorylated N is found in both the cytoplasm and the nucleolus. Furthermore, the serine/threonine kinase inhibitors, H-7 and sangivamycin, have only a limited effect on the localization of N-EGFP (unreported results).

The localization of HIV Rev to the nucleus/nucleolus is regulated by RNA binding (Ernst et al., 1997). In the presence of transcription inhibitors such as actinomycin D, nucleo-cytoplasmic shuttling is interrupted resulting in the accumulation of viral protein in the cytoplasm (Pinol-Roma and Dreyfuss, 1992; Soliman and Silverstein, 2000). However, the addition of 20 μg/ml actino-

mycin D to cells expressing N-EGFP produces the opposite effect, i.e. the accumulation of PRRSV N protein in the nucleus (data not shown), which suggests that RNA binding may regulate both N protein assembly (Wootton and Yoo, 2003) as well as localization. Further experiments are required to determine the roles of RNA binding, phosphorylation and dimer formation in the regulation of N protein localization.

8. Why does PRRSV N protein localize to the nucleolus?

Even though the structure and function of coronavirus and arterivirus N proteins have been the subject of intense study for several years, the localization of the N protein to the nucleus/nucleolus has come to light very recently (Rowland et al., 1999; Wurm et al., 2001). The function of N protein in the nucleolus presents at least three possibilities. First, lysine/arginine-rich RNA binding domains can mimic nuclear and nucleolar localization signal sequences (LaCasse and Lefebvre, 1995). Overproduction of N protein combined with a mechanism for export from the nucleus would ensure that sufficient quantities of N protein would be available for incorporation into Ns. A second and closely related possibility is that nucleolar accumulation of N is a host cell defense strategy designed to inhibit viral assembly. Since PRRSV assembles viral proteins in the ER–Golgi compartment, the diversion of large quantities of N protein to the nucleolus would deprive the replicating virus of an essential structural protein. As a counter-strategy, the virus would either overproduce N or incorporate an NES to return N to the cytoplasm. And finally, a third possibility is that nucleolar localiza-

tion of N participates in the modulation of nucleolar function, a strategy designed to optimize virus replication. Besides its function in rRNA synthesis and ribosome assembly, the nucleolus participates in the regulation of several host cell processes, including regulation of the cell cycle, apoptosis and the induction of antiviral responses. Therefore, the effect of N on nucleolar function would be through interactions with nucleolar proteins, rRNA or rDNA. Chen et al. (2002) showed that a coronavirus N protein was involved in the redistribution of the nucleolar protein, fibrillarin, and was able to form an interaction with another nucleolar protein, nucleolin. The eventual effect of N protein expression was the inhibition of cell division. Decreased proliferation would benefit the virus by diverting biosynthetic resources from the dividing nucleus to the cytoplasm, the site of coronavirus replication. Presumably, the inhibition of cell proliferation is occurring by the direct interaction of N protein with fibrillarin, nucleolin and other nucleolar proteins. It remains to be seen if the N protein of PRRSV behaves in a similar manner.

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