Full Length Research Paper

Characterisation of human coronavirus-NL63 nucleocapsid protein

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Coronavirus N is a multifunctional protein that plays an essential role in enhancing the efficiency of virus transcription and assembly. This manuscript reports the analysis of HCoV-NL63 N protein by comparing the amino acid sequences of coronavirus N-homologues. A ~50 kDa protein was expressed in both a mammalian cell and bacterial cell system that is similar in size to the predicted ~42.6 kDa HCoV-NL63 N protein. PSORTII identified two putative nuclear localisations signals and PONDR identified one disordered region in HCoV-NL63 N. The reported protein analysis serves as a prelude to laboratory analysis to understand the processing of HCoV-NL63 N.

Key words: HCoV-NL63, nucleocapsid protein, protein analysis, nuclear localisation signals, protein ordered/disordered regions.

INTRODUCTION

Genome sequencing technologies have resulted in large genome databases and the rate of genome sequencing has surpassed our ability to determine the function of the proteins these genomes encode. *In silico* analysis provides tools to predict possible functions of these proteins by comparing novel proteins of unknown function with proteins of known function, or by analyzing the unknowns using an algorithm based on information from proteins of known function. Thus, the theoretical analysis of proteins provide a useful framework that has given researchers the ability to predict the function of these proteins, which can then be verified with the use of *in vitro* and *in vivo* studies (MacKenzie et al., 2008; Mahotka et al., 2002). Coronaviruses are enveloped viruses of the *Coronaviridae* family in the order *Nidovirales*. Recently, the International Committee for Taxonomy of Viruses (ICTV) reported that the three traditional coronavirus groups are replaced by four genera, namely *Alphacoronaviruses*, *Betacoronaviruses*,

Gammacoronaviruses and *Deltacoronaviruses* (http://talk.ictvonline.org/files/ictv_documents/m/msl/4090.aspx). Coronaviruses have single-stranded, positive sense RNA genomes, ranging in size from 25 to 30 kbs.The genomes typically encode for four structural proteins, spike (S), envelope (E), membrane (M) and nucleocapsid (N). The coronavirus N protein is one of the most abundantly produced viral proteins within an infected cell. This protein has been shown to have several functions, including binding to viral RNA to form the ribonucleocapsid and is proposed to have roles in replication, transcription and translation. To date, coronavirus N proteins have been shown to cause deregulation of the host cell-cycle (Surjit et al., 2006; Wurm et al., 2001), inhibit the production of interferon (Kopecky-Bromberg et al., 2007), up-regulate the production of COX2 (Yan et al., 2006), up-regulate AP1 activity (He et al., 2003), induce apoptosis in serum starved COS1-cells (Surjit et al., 2004a) and interact with various cellular proteins including cyclophilin A (Luo et al., 2004) and hnRNP-A1 (Luo et al., 2005; Wang and Zhang, 1999), which may have possible pathological consequences.

Even though several studies have shown that the N-

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protein of members of Nidovirales localizes to the nucleus and/or nucleolus (Cawood et al., 2007; Hiscox et al., 2001; Timani et al., 2005; Wurm et al., 2001), others have shown that N localizes predominantly or exclusively to the cytoplasm (Rowland et al., 2005; Stohlman et al., 1983; You et al., 2005). This indicates that the localization of N to the nucleus or nucleolus is not a conserved property of nidoviruses (Rowland et al., 2005). The N-protein of coronaviruses is highly antigenic and reliably induces an antibody response (Mourez et al., 2007) and has therefore been recognized as an important target in the development of coronavirus diagnostics (Blanchard et al., 2011; Lee et al., 2008).

HCoV-NL63 was first isolated from the nasopharyngeal aspirate of a 7 month old infant from Holland (van der Hoek et al., 2004). The virus has since been shown to have a global distribution and is observed primarily in the winter season in temperate climates. To date, the virus has been associated with acute respiratory illness and croup in young children, the elderly and immunocompromised individuals (Oosterhof et al., 2010; Sung et al., 2010; van der Hoek et al., 2005, 2006). Clinical manifestations most frequently associated with HCoV-NL63 infections include cough, coryza, fever, sore throat, bronchiolitis, bronchitis, croup and pneumonia (Fielding, 2011; van der Hoek, 2007). To date, three groups have reported a fatal outcome following HCoV-NL63 infection, indicating that this virus could be involved in more severe respiratory tract disease (Bastien et al., 2005; Cabeca and Bellei, 2012; Oosterhof et al., 2010); all of these cases had underlying suppressed or compromised immune systems.

Unfortunately, the virus is not well studied at a molecular level with only the spike (Lin et al., 2008, 2011; Mathewson et al., 2008) and ORF3 proteins (Fielding and Suliman, 2009; Muller et al., 2010) previously characterized. In this work, the HCoV-NL63 N was expressed in mammalian and bacterial systems. Various bioinformatics tools were used to analyze the HCoV-NL63 N amino acid sequence. The importance of identified putative nuclear localization signals and disordered motifs were discussed. This manuscript reports the analysis of HCoV-NL63 N as a prelude to the molecular characterization of N in *in vitro* mammalian and bacterial cell systems.

RESULTS AND DISCUSSION

The HCoV-NL63 genome is approximately 27 553 nucleotides in size, with the typical coronavirus genome organization. It produces six separate mRNAs, with all potential ORFs encoding for viral proteins. N is expressed from distinct subgenomic (sg) mRNA 6, the most abundant sg mRNA (Pyrc et al., 2004). To determine the size of HCoV-NL63 N, recombinant GST-N was expressed in a bacterial system, purified by affinity column and treated with TEV to remove the GST-tag, according to the manufacturer's specification (Promega). Also, recombinant HCoV-NL63 N-HA was transfected and expressed in Cos-7 cells (Khan et al., 2006). SDS-PAGE analysis of purified bacterial expressed protein (Figure 1A) and Western Blot analysis of total mammalian cell lysate (Figure 1B) showed ~50 kDa proteins which is larger than the size of the 42.6 kDa protein predicted by our initial analysis (http://www.sciencegateway.org/tools/ proteinmw.htm). This discrepancy in size showed that the protein was probably pre-, co- or post-translationally modified; the type of modification is currently being investigated.

Next, the amino acid sequence of HCoV-NL63 N was compared to other coronavirus N proteins (Table 1). HCoV-NL63 was shown to be most similar to the human coronavirus 229E N (43% identity; 63% similarity). This was not totally unexpected, since previous molecular clock analysis estimate that HCoV-NL63 and HCoV-229E evolved from a most recent common ancestor in the 11th century (Pyrc et al., 2006). Interestingly, HCoV-NL63 N also showed high amino acid similarity to the bat coronavirus HKU7 N (40% identity; 57% similarity). As speculated previously (Fielding and Suliman, 2009), this high amino acid similarity raises interesting questions about the relationship between this human and bat coronaviruses. Woo and colleagues postulate that the ancestor of all present-day coronaviruses infected a bat and then jumped from that bat to a bird, or alternatively, first infected a bird and then jumped to a bat, evolving dichotomously. This bat coronavirus then jumped to another bat species, which in turn jumped to other bat species or other mammals, including humans. Each of these interspecies jumps resulted in the independent evolution of these coronaviruses and gave rise to the current coronavirus genera. In fact, present evidence suggests that the huge diversity of coronaviruses in bats make them excellent gene pools for group 1 and group 2 coronaviruses (Woo et al., 2009).

Comparative analysis using CLUSTAL X version 2.0 (Larkin et al., 2007) of 10 coronavirus species identified a 9 amino acid region conserved between the N homologues, that is FYY(L/T)GTGP (Figure 2). Conserved regions within a family of proteins is commonly indicative of structural and functional conservation, as well as an evolutionary relationship. The function of this sequence, if any, is yet to be elucidated. Since interactions between coronavirus RNA and N are assumed to predominantly take place at the nucleocapsid N-terminal domain, the possible role of this conserved motif in RNA-N interaction will be studied. However, several other binding sites within N have also previously been identified, indicating that both N- and C-terminal domains are probably involved in RNA binding (Chang et al., 2009).

Translocation of proteins from the cytoplasm through the nuclear pore complex and into the nucleoplasm is
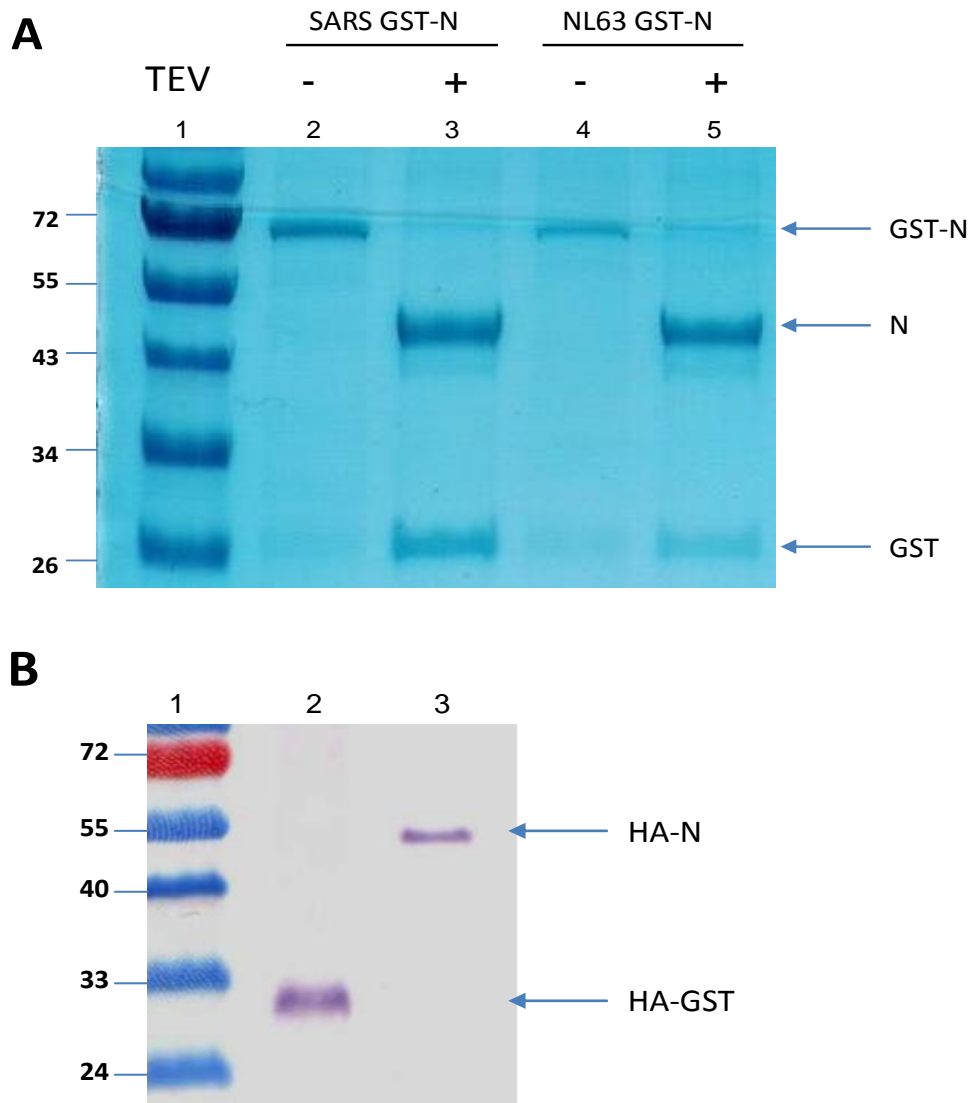
Figure 1. Expression of HCoV-NL63 N in: (A) a bacterial cell system. Cleavage of the GST-tag from SARS-CoV and HCoV-NL63 full length N using ProTEV protease (Promega). SARS-CoV N and NL63 N were reverse transcribed from viral RNA and PCR amplified using nucleocapsid specific primers. The N-gene was cloned into the pFlexi™ protein expression vector (Promega), which appends an N-terminal GST affinity tag. Protein expression was induced by addition of 0.1% Rhamnose (Promega). Lane 1: Pre-stained molecular weight protein marker; Lane 2: SARS-CoV GST-N; Lane 3: SARS-CoV N with GST cleaved; Lane 4: HCoV-NL63 GST-N; Lane 5: HCoV-NL63 N with GST cleaved. SARS-CoV N was included as a control. (B) a mammalian cell system. Recombinant plasmid pCAGGS-N-HA (HCoV-NL63 N gene cloned into vector pCAGGS which appends an N-terminal Haemagglutinin tag to expressed protein) was transfected with Lipofectamine 2000 (Invitrogen) into a COS-7 cell line (African green monkey cells) and grown at 37°C in 5% CO_2 for 16 h. Cells were then lysed and the target protein detected on a Western Blot using rabbit anti-HA polyclonal (1:250) as primary antibody and goat anti-Rabbit polyclonal secondary antibody (1:1000).

initiated by nuclear localization signals (NLSs) on the protein binding to a NLS-binding site on importin-α of the host cell (Rowland et al., 2005). NLSs are rich in basic amino acids and conform to one of three types, that is, (i) monopartite pat4, (ii) monopartite pat7 or (iii) bipartitemotifs. Pat4 NLSs are recognized by the presence of 4 basic residues - either K or R or the presence of 3 basic residues (K/R) and/or (H/P). Pat7 NLSs are segments beginning with P and followed within 3 residues by a basic segment containing 3 K or R residues out of 4. On the other hand, bipartite NLSs begin with 2 basic residues, followed by a 10 residue

Table 1. Comparison of HCoV-NL63 N amino acid sequence to homologues from selected coronavirus isolates. Identity values (%) are shown in BOLD and similarity values (%) are shown in *ITALICS*.

Coronavirus	SARS	MHV	IBV	OC43	229E	TGEV	PEDV	HKU1	HKU7
NL63	**21**	**18**	**17**	**18**	**43**	**32**	**35**	**20**	**40**
	34	*31*	*31*	*32*	*63*	*51*	*50*	*34*	*57*

To identify homologues, the amino acid sequence of HCoV-NL63 N was compared to sequences in the GenBank database at the National Centre for Biotechnology by using the Basic Blast Search Server (Altschul et al., 1990). Subsequently, HCoV-NL63 N was aligned with selected coronavirus N proteins with CLUSTAL X version 2 (Larkin et al., 2007). The sequences aligned, with Genbank accession numbers in bold, were: HCoV-NL63: NL63 (DQ846901.1); SARS-CoV: SARS (AY360146.1); Mouse Hepatitis Virus: MHV (AAU06361); Infectious Bronchitis Virus: IBV (AAA46214), Human coronavirus OC43: HCoV-OC43 (AY585229.1); HCoV-229e: 229E (AAG48597); Transmissible gastroenteritis virus: TGEV (AAA47915); Porcine epidemic diarrhea virus:PEDV (AF353511); Human coronavirus HKU1: HKU1 (AAT98585) and Bat coronavirus HKU7: HKU7 (DQ666343).
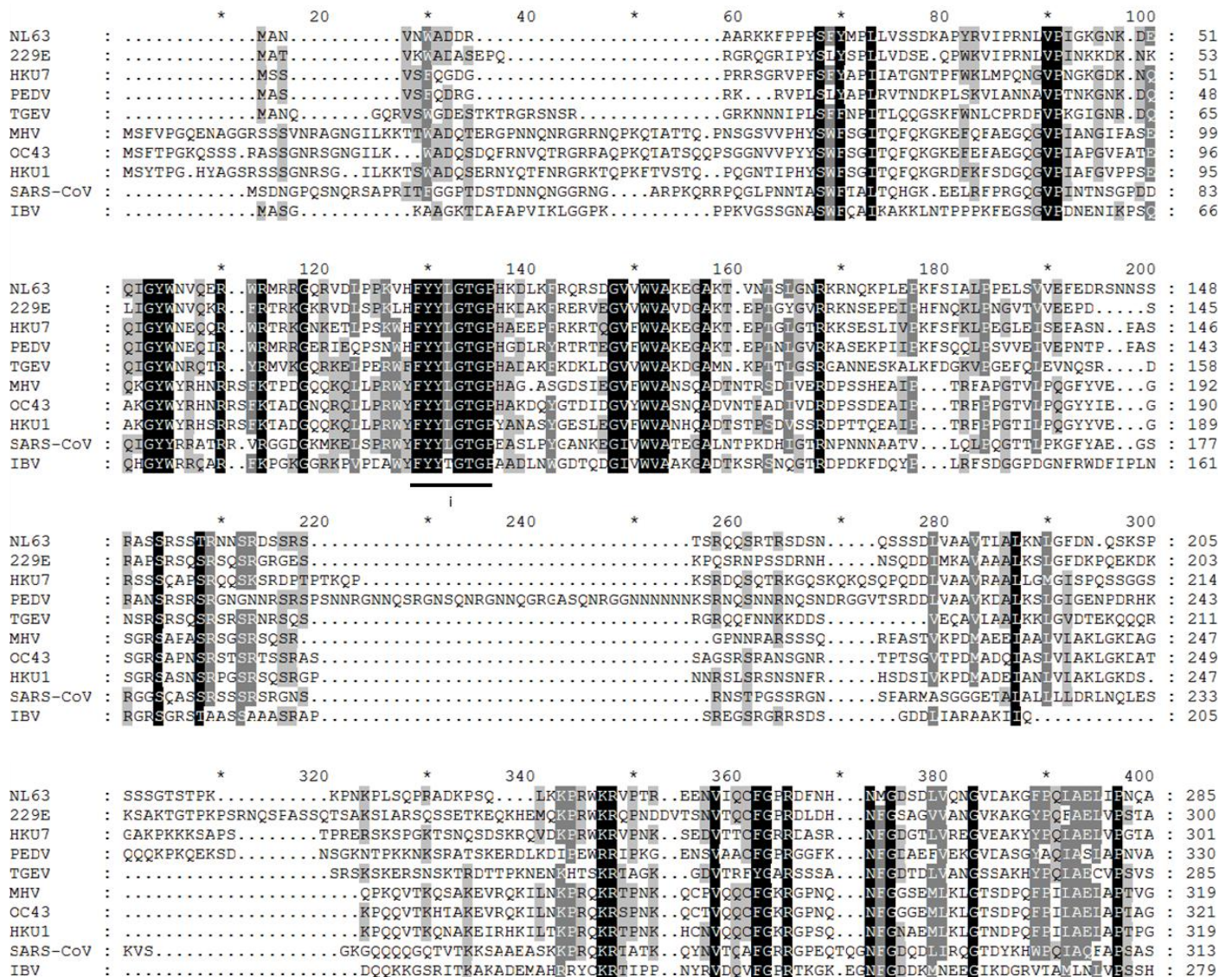

Figure 2. Comparative analyses of HCoV-NL63 N. Selected HCoV-NL63 N-homologues were aligned with CLUSTAL X version 2.0 (Larkin et al. 2007) and viewed with GENEDOC version 2.6.002 software (Nicholas et al; 1997). Shading indicates conserved regions and gaps were introduced to align sequences. Conserved region identified: (i) FYY(L/T)GTGP. N amino acid sequences were obtained from NCBI (accession numbers in brackets): HCoV-NL63 (DQ846901.1), SARS-CoV (AY360146.1), MHV (AAU06361), IBV (AAA46214), HCoV-OC43 (AY585229.1), HCoV-229E (AAG48597), TGEV (AAA47915), PEDV (AF353511), HKU1 (AAT98585) and HKU7 (DQ666343).

Figure 2. Continued.

spacer, which is then followed by another basic region composed of 3 basic residues out of 5 residues. PSORT II (http://www.genscript.com/psort/psort2.html) identified two possible NLSs for HCoV-NL63 N. A predicted pat4 motif was identified at amino acid position 232-235 (KKPR) and a predicted pat7 motif at position 234-240 (PRWKRVP); no bipartite motifs were identified for NL63 N-protein.

Predictors of Natural Disordered Regions (PONDR) was used to predict possible disordered regions within HCoV-NL63 N. PONDR (http://www.pondr.com) uses several algorithms, including VL-XT, XL1-XT, VL3 and VSL1 to predict possible disordered regions. In this study, the VL-XT and VSL1 algorithms were used. VL-XT incorporates 3 feed-forward neural networks which are based on long disordered regions characterized by x-ray chrystallographic data (Li et al., 1999; Romero et al., 1997, 2001). The VSL1 utilizes two algorithms for short (< 30aa) and long (> 30aa) disordered regions and is trained on 1335 protein sequences (Obradovic et al., 2005). Even though *in silico* analysis is a good predictor of protein structure, errors can occur and findings must be substantiated with experimental evidence. The order/disorder state of HCoV-NL63 N was different to that of other coronavirus N-proteins, with HCoV-NL63 N having only one disordered region (residues 109 to 248) compared to the 2 to 3 disordered regions within other coronavirus N-proteins (Chen et al., 2005). Interestingly, many of the motifs and/or regions important for the interaction of SARS-CoV N with other proteins fall within these disordered regions (He et al., 2004; Huang et al., 2004; Luo et al., 2004; Surjit et al., 2004b). For this reason, residues 109 to 248 of HCoV-NL63 N could be crucial for interactions between HCoV-NL63 and other proteins.

In this study, molecular and bioinformatics tools were used to analyse HCoV-NL63 nucleocapsid protein by comparing it to selected coronavirus N proteins. Putative motifs that could be essential for the functioning of this protein have been identified; these are currently being verified in our laboratory. To our knowledge, this is the first manuscript reporting the molecular analysis of HCoV-NL63 N.

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