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Short communication

SARS-coronavirus protein 6 conformations required to impede protein import into the nucleus

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The severe acute respiratory syndrome coronavirus (SARS-CoV) genome encodes eight accessory proteins. Accessory protein 6 is a 63-residue amphipathic peptide that accelerates coronavirus infection kinetics in cell culture and in mice. Protein 6 is minimally bifunctional, with an N-terminal lipophilic part implicated in accelerating viral growth and a C-terminal hydrophilic part interfering with general protein import into the nucleus. This interference with nuclear import requires interaction between protein 6 and cellular karyopherins, a process that typically involves nuclear localization signal (NLS) motifs. Here we dissected protein 6 using site-directed mutagenesis and found no evidence for a classical NLS. Furthermore, we found that the C-terminal tail of protein 6 impeded nuclear import only in the context of a lipophilic N-terminus, which could be derived from membrane proteins unrelated to protein 6. These findings are discussed in the context of the proposed protein 6 structure.

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The coronaviruses (CoVs) are enveloped, plus-strand RNA viruses that are widespread in nature, infecting birds as well as land, sea and flying mammals, causing humans gastrointestinal and respiratory diseases of varying severity. The ~30 kb CoV RNA genomes are all distinguished by conserved gene organizations. The CoVs encode well-described proteins required for virus entry, replication and assembly, and also encode more enigmatic small proteins of unknown function that are specific to one of the three CoV antigenic groups (Stadler et al., 2003). Reverse genetic engineering methods have been used to ablate one or more of these smaller group-specific genes, and in general, these engineered manipulations have had little effect on in vitro CoV viabilities (Yount et al., 2005). These fundamental observations make it clear that CoVs encode “accessory” proteins that are unnecessary for virus amplification in cell culture but are presumably operating in more complex in vivo environments to maintain these viruses in nature. Experiments to discern CoV accessory protein structure and function may contribute generally to CoV virulence and zoonotic potential, and their genetically engineered modification may be required to construct useful CoV vectors and attenuated CoV vaccines.

SARS-CoV is the prototypic human pathogenic CoV. SARS-CoV is also the most complex known CoV with respect to accessory proteins, with eight accessory genes interspersed between those encoding virion structural components (Hussain et al., 2005; Rota et al., 2003; Snijder et al., 2003). These accessory genes are present in SARS-CoVs isolated from bats, civet cats, raccoon dogs, and humans, suggesting important accessory functions in a variety of host environments (Li et al., 2005; Wang et al., 2005). There is clear evidence that these accessory genes are expressed (Chen et al., 2007; Keng et al., 2006; Lu et al., 2006; Nelson et al., 2005; Pewe et al., 2005; Schaecher et al., 2007a,b; Tan et al., 2004; Yuan et al., 2006, 2005) but their functions remain somewhat obscure. Currently there are a range of suggested and indicated activities for accessory proteins, including viral structural elements, ion channels, cell death inducers and interferon antagonists (Chen et al., 2007; Frieman et al., 2007; Hussain et al., 2008; Kopecky-Bromberg et al., 2007; Lu et al., 2006; Schaecher et al., 2007a,b; Tan et al., 2004; Yuan et al., 2005).

Of all the CoV accessory proteins currently under investigation, the SARS-CoV protein 6 is arguably the best understood with respect to its structure and function. This intriguing 63 amino acid peptide is amphipathic in its amino-terminal ~44 residues and very polar in the ~20 residues comprising its C-terminus. Protein 6 is found entirely on cytoplasmic membranes (Geng et al., 2005), localizing on ER and Golgi organelles. Its membrane topology is N-endo C-endo, with a membrane-embedded stretch that is likely ~6 nm of alpha-helical structure (Netland et al., 2007; Zhou et al., 2010). With respect to function, protein 6 is a virulence factor, converting a sub-lethal infection of heterologous murine coronavirus into a lethal infection and conferring growth advantages to murine coronavirus and also to SARS-CoV in infected host mice and cultured cells (Netland et al., 2007; Zhao et al., 2009). The way that protein 6 supports these CoV infections may be two-fold. The N-terminal lipophilic portion, presumably by remodeling
intracellular membrane architectures, supports more robust CoV RNA replication (Pewe et al., 2005; Tangudu et al., 2007). The C-terminal polar portion, by interfering with protein import into the nucleus, detains transport of signaling proteins needed for innate immune responses (Frieman et al., 2007; Kopecky-Bromberg et al., 2007). The way that protein 6 interacts with nuclear import factors appears to be similar to that of a classical NLS-containing protein, however, motifs and domains involved in the communication between protein 6 and nuclear import machineries are not fully characterized.

A classical NLS is (1) necessary for nuclear import, (2) sufficient to target an unrelated protein to the nucleus, (3) able to interact with its putative import receptor.

Previous studies have shown that the C-terminus of SARS-CoV accessory protein 6 binds to karyopherin-α2 (KPNA2) (Frieman et al., 2007), and KPNA2 binds to karyopherin-β (KPNB1) (Gorlich et al., 1995). These protein complexes are restricted to cytoplasmic membranes via the hydrophobic N-terminus of protein 6, which limits capacities of KPNA2 and KPNB1 to escort proteins from the cytoplasm to nuclei (Moroianu et al., 1995). KPNA2 typically binds and directs the transport of proteins containing the “classical” nuclear localization signal (cNLS) (Moroianu et al., 1995), which is lacking in protein 6. Protein 6 does, however, contain a cluster of four basic residues at its C-terminus, which weakly resembles a bipartite NLS (Kalderon et al., 1984). To assess whether this putative NLS is necessary for p6 to impede protein import into nuclei, we replaced basic residues (RKKK) to alanine by PCR based mutagenesis (Fig. 1). If these basic residues are part of a KPNA2-interacting NLS motif, then their replacement with alanines would eliminate p6-mediated interference with nuclear protein import. Co-expression of mutant p6RKKK-ala, with a cNLS bearing reporter (3XGFP-NLS) revealed that the mutant protein lacking basic residues was as potent as native p6 in blocking nuclear import of the cNLS reporter (Fig. 1 and Table 1). These data indicate that the potential bipartite NLS located at protein C-terminus does not play a role in preventing nuclear import. Interaction between protein 6 and import factors may therefore depend on protein conformation or may be mediated by an unknown NLS-containing adaptor protein.

A conformation-dependent NLS on protein 6 might demand that the entire C-terminus be presented to components of the nuclear import machinery. To identify the minimal required C-terminal residues comprising this putative NLS, chimeric protein constructs were prepared. Each construct contained an N-terminal hydrophobic region (amino acid residues 1–30) derived from Hepatitis C Virus Non-Structural protein 5A (HCV NS5A) and C-terminal segments of variable length (amino acid residues 45–63, 35–63 or 24–63) derived from protein 6. Both the HCV NS5A and the protein 6 N-termini are ER membrane-associated (Brass et al., 2002) but bear no primary sequence similarities. This makes it likely that the chimeric proteins occupy subcellular positions that are similar to those of native protein 6, but at the same time, permit the

### Table 1

| Protein | Cytoplasmic | Nuclear |
|---------|-------------|---------|
| p6      | 80 ± 3.86   | 19 ± 3.86 |
| P6-ala  | 79 ± 3.38   | 20 ± 3.38 |
| Vector  | 25 ± 5.28   | 74 ± 5.28 |
| E-p634–63 | 21 ± 3.05 | 78 ± 3.05 |
| E-p635–63 | 20 ± 6.56 | 79 ± 6.56 |
| E-p645–63 | 25 ± 5.17 | 74 ± 5.17 |
| N55A-p634–63 | 77 ± 2.44 | 22 ± 2.44 |
| N55A-p635–63 | 15 ± 1.51 | 84 ± 1.51 |
| N55A-p645–63 | 18 ± 4.30 | 81 ± 4.30 |

*Fig. 1. Mutagenic analysis of the potential NLS on protein 6. Protein 6 contains an N-terminal amphipathic region and a C-terminal signal rich region containing a cluster of four basic residues (bold italics). To determine the role of C-terminal basic residues in nuclear import, the indicated R–K–KK were substituted to alanine by overlapping PCR. 293 cells were co-transfected with indicated plasmids (left column) and at 24 h post-transfection, intracellular distributions of GFP-NLS reporters were determined by laser scanning confocal microscopy.*
evaluation of nuclear import independently from other proposed functions associated with the N-terminal amphipathic region of p6 (Netland et al., 2007).

To determine the effect of chimeric NSSA-p6 proteins on nuclear import of cNLS bearing reporter, NSSA-p6 proteins (NSSA-p624–63, NSSA-p635–63 and NSSA-p645–63) were individually expressed in concert with the cNLS-GFP reporter. Intracellular localization of the reporter protein was then determined by immunofluorescence microscopy. The NSSA-p6, all C-terminally tagged with HA epitopes, were also visualized by western blot and immunofluorescence microscopy.

As expected, intracellular localization of NSSA-p6 fusion proteins was similar to native protein 6, colocalizing both with Golgi and ER markers (Fig. 2A). Nuclear accumulation of reporter protein was greatly reduced in cells transfected with the NSSA-p624–63 construct, but not in the NSSA-p635–63 and NSSA-p645–63 – expressing cells (Fig. 2B and Table 1). Protein 6-mediated nuclear import inhibition depends on a threshold p6 accumulation, which is necessary for the partial or complete titration of cytoplasmic importin-β. Western blot analyses revealed an abundant and comparable level of expression of all three of the chimeric p6 proteins in transfected cells (Fig. 2B, bottom panel), indicating that the observed differences in nuclear import impedance were not simply due to differences in protein accumulation. These results thus suggested that the region of protein 6 that is sufficient to impede nuclear import extends beyond the known required residues 52–63 (Frieman et al., 2007; Hussain et al., 2008).

Previously published reports have implicated the C-terminal residues 52–63 in p6 interaction with karyopherin-α2 and nuclear import inhibition. Our results further extended these findings and revealed that residues 52–63 are part of the larger functional 24–63 domain that is necessary for nuclear import obstruction. The sequence analysis and mutational studies indicated that protein 6 does not contain a classical sequential NLS and that p6 interactions with nuclear import machinery are most likely conformation-dependent. A conformation-dependent NLS might demand that the entire C-terminus be presented to components of the nuclear import machinery and might also be extremely sensitive to inactivation by any of a number of substitution or deletion mutations.

To determine whether the p6 C-terminal sequences can target an unrelated cytosolic protein to the nucleus, a set of 3XGFP constructs were appended with p6 residues 24–63, 35–63 and 45–63. The 84 kDa 3XGFP protein exceeds the passive diffusion limit of an unrelated cytosolic protein to the nucleus. A set of 3XGFP constructs were appended with p6 residues 24–63, 35–63 and 45–63. The 84 kDa 3XGFP protein exceeds the passive diffusion limit of an unrelated cytosolic protein to the nucleus. A set of 3XGFP constructs were appended with p6 residues 24–63, 35–63 and 45–63. The 84 kDa 3XGFP protein exceeds the passive diffusion limit of an unrelated cytosolic protein to the nucleus.

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Fig. 2. Nuclear import inhibition and cellular distribution of NS5A-p6 fusion proteins. (A) 293 cells were transiently transfected with indicated plasmids (top panel) and 24 h later, colocalization of HA-tagged fusion proteins with Golgi (upper panel: golgin 97, green; protein 6, red) and endoplasmic reticulum (lower panel: ER marker (DsRed-ER), red; protein 6, green) was determined by laser scanning confocal microscopy. (B) 293 cells were transiently transfected with p6 or with the indicated NS5A-p6 fusion proteins (top panel) and with reporter expression plasmid 3XGFP-SV40NLS. 24 h later, intracellular distributions of recombinant proteins (red) and GFP (green) were determined by laser scanning confocal microscopy. Accumulations of indicated proteins in transfected cells were determined by WB using anti-HA antibody (bottom panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 3. Effects of protein 6 C-terminal appendages on soluble and membrane proteins. (A) 293 cells were transiently transfected with HA-tagged 3XGFP-p6 encoding plasmids. 24 h later, nucleocytoplasmic distributions of GFP were determined by fluorescent microscopy and expression of full-length fusion proteins was determined by western blotting using anti-HA and anti-GFP antibodies. (B) 293 cells were co-transfected with plasmids encoding HA-tagged full-length p6, E or E-p6 fusion protein and 3XGFP-NLS reporter plasmids. 24 h post-transfection, cells were fixed, stained with anti-HA antibody and cellular distribution of fusion proteins (red) and GFP reporters (green) was determined by laser scanning confocal microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Herpesvirus 1 Tegument Protein VP22 accumulates in the nucleus of infected cells but does not contain a classical sequential NLS. A relatively long 103 amino acid sequence is required for nuclear localization (Zhu et al., 2005). The human cytomegalovirus (HCMV) protein UL84 (pUL84) utilizes the importin-α/β pathway but does not contain a classical importin-α binding motif, rather a large domain of ~282 residues is necessary for nuclear localization (Lischka et al., 2003). Several cellular nuclear-cytoplasmic shuttling proteins also lack well defined nuclear localization or nuclear export signals. The tumour suppressor PTEN lacks obvious canonical nuclear localization signal (NLS) sequences or nuclear export sequences (NES) that could account for its targeting (Gil et al., 2007). The latent transcription factor STAT1 which is activated in response to IFN-γ and translocated into the nucleus does not contain a classical NLS as defined by the basic amino acid cluster. Rather, interaction with nuclear import machinery is unconventional, dependent on tyrosine phosphorylation and homodimerization (Sekimoto et al., 1997). Thus a conformation-dependent motif facilitating cytoplasmic – nuclear protein transport is often apparent. An interesting feature of the SARS-coronavirus protein 6 is that it presents such a motif to impede general nuclear import.

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