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Characterization of the spike protein of human coronavirus NL63 in receptor binding and pseudotype virus entry

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ABSTRACT

The spike (S) protein of human coronavirus NL63 (HCoV-NL63) mediates both cell attachment by binding to its receptor hACE2 and membrane fusion during virus entry. We have previously identified the receptor-binding domain (RBD) and residues important for RBD–hACE2 association. Here, we further characterized the S protein by investigating the roles of the cytoplasmic tail and 19 residues located in the RBD in protein accumulation, receptor binding, and pseudotype virus entry. For these purposes, we first identified an entry-efficient S gene template from a pool of gene variants and used it as a backbone to generate a series of cytoplasmic tail deletion and single residue substitution mutants. Our results showed that: (i) deletion of 18 aa from the C-terminus enhanced the S protein accumulation and virus entry, which might be due to the deletion of intracellular retention signals; (ii) further deletion to residue 29 also enhanced the amount of S protein on the cell surface and in virion, but reduced virus entry by 25%, suggesting that residues 19–29 contributes to membrane fusion; (iii) a 29 aa-deletion mutant had a defect in anchoring on the plasma membrane, which led to a dramatic decrease of S protein in virion and virus entry; (iv) a total of 15 residues (Y498, V499, V531, G534, G537, D538, S540, G575, S576, E582, W585, Y590, T591, V593 and G594) within RBD were important for receptor binding and virus entry. They probably form three receptor binding motifs, and the third motif is conserved between NL63 and SARS-CoV.

1. Introduction

Human coronavirus NL63 (HCoV-NL63, NL63) was first described in 2004 in the Netherlands (van der Hoek et al., 2004). However, it was already isolated in 1988 (Fouchier et al., 2004), or even earlier (van der Hoek et al., 2006). NL63 has since been reported in more than 15 countries across the world and accounts for up to 9.3% of acute respiratory infections leading to hospitalization (Albuquerque et al., 2009; Arden et al., 2005; Bastien et al., 2005; Chiu et al., 2005; Dare et al., 2007; Ebihara et al., 2005; Esper et al., 2005; Gerna et al., 2006; Han et al., 2007; Koetz et al., 2006; Moes et al., 2005; Oosterhof et al., 2009; Smuts et al., 2008; Vabret et al., 2005). These findings suggest that NL63 has been widely circulating in the human population for decades. NL63 infection generally causes mild upper respiratory tract diseases, but may also cause more severe lower respiratory tract diseases, e.g. croup, bronchiolitis, and pneumonia in young children, the elderly, and immunocompromised people (van der Hoek et al., 2006). No vaccine or antiviral drug is currently available for NL63.

Coronaviruses can be divided into three groups based on antigenic and phylogenetic relationships. NL63 belongs to group I (van der Hoek et al., 2004). Like other coronaviruses, the 5′ proximal two-third of NL63 genome is occupied by two large replicase genes 1a and 1b, while the 3′ proximal one-third encodes four structural protein genes: spike (S), envelope (E), membrane (M), and nucleocapsid (N). These genes are arranged in a conserved order 5′-1a-1b-S-E-M-N-3′ (Fig. 1).

The S protein of coronaviruses forms a layer of long petal-shaped surface spikes that give the virions a crown-like appearance when visualized by electron microscopy (Lai and Holmes, 2001). S protein is anchored on the viral envelope, a lipid bilayer derived from the intracellular membrane during virus budding. S protein is a type I transmembrane protein containing a signal peptide, long N-terminal ectodomain, transmembrane (TM) domain, and a short C-terminal cytoplasmic tail (Fig. 1). The signal peptide directs the

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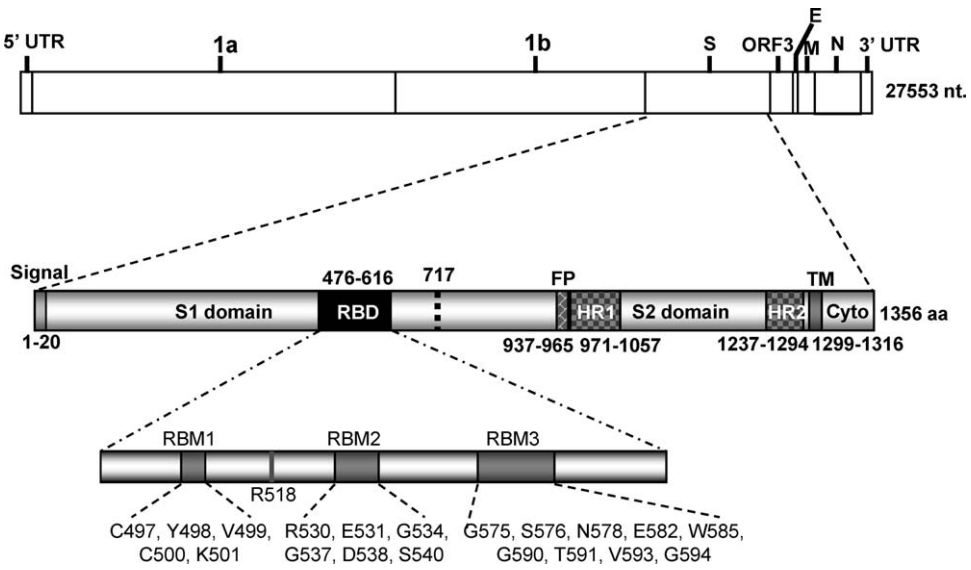

Fig. 1. Schematic diagram of genome organization of NL63 and domain architecture of spike (S) protein. Signal: signal peptide; RBD: receptor-binding domain; FP: fusion peptide; HR: heptad repeat; TM: transmembrane domain; Cyto: cytoplasmic tail. Twenty-one residues that have been previously shown to be important for RBD–hACE2 interaction are indicated within three receptor binding motifs (RBMs) (Li et al., 2007; Lin et al., 2008).

nascent S polypeptide to the endoplasmic reticulum (ER), where the signal peptide is cleaved and monomeric precursors are synthesized. The precursors are heavily glycosylated to yield 150–200 kDa mature monomers, which are further oligomerized into trimers within the ER (Masters, 2006). S trimers interact with M protein through their cytoplasmic tails and are transported to the ER–Golgi intermediate compartment (ERGIC) where assembly and budding occur (Hogue and Machamer, 2008). This is in contrast to the assembly and budding of many enveloped viruses, e.g. HIV, that occur on the plasma membrane (Chen and Lamb, 2008).

In groups 2a, group 3 and some group 1 coronaviruses, the S proteins are cleaved in the Golgi complex by a furin-like protease into noncovalently associated S1 and S2 subunits prior to assembly into the virion (de Haan et al., 2008; de Haan and Rottier, 2005). The S protein of NL63 does not have furin-recognition site and is thus not cleaved during biogenesis. Nevertheless, the S1 (21–717 aa) and S2 (718–1356 aa) domains can still be deduced based on sequence similarity.

The S1 domain of coronavirus mediates cell attachment through interaction with a cellular receptor. As such, the particular S1–receptor interaction largely determines the host range, tissue and cell tropisms of coronaviruses. In addition, S1 is the major determinant for eliciting neutralizing antibody, which in turn blocks virion–receptor binding and protects animals from infection (Gallagher and Buchmeier, 2001; Wentworth and Holmes, 2007). Many coronaviruses have variations in S1 that evade immune response. Amazingly, variations as small as one amino acid have been shown to change receptor usage, tissue tropism and pathogenesis (Ballesteros et al., 1997; Krempl et al., 2000). The S2 domain mediates membrane fusion during viral entry into host cells. It contains several conserved regions characteristic of class I viral fusion proteins: fusion peptide (FP), two heptad repeats (HRs), TM domain and several conserved cysteine residues in the cytoplasmic tail (Fig. 1). Each of these structural features has been shown to contribute to membrane fusion (Wentworth and Holmes, 2007).

The primary entry receptor for NL63 was identified as human angiotensin-converting enzyme 2 (hACE2), which is also the primary receptor for group 2b SARS-CoV. This is particularly interesting since the S proteins of these viruses share only 21% amino acid identity (Hofmann et al., 2005; Li et al., 2003). Although they use the same receptor for entry, these two viruses cause different clinical outcomes. In contrast to NL63 that generally causes mild respiratory diseases, such as croup in children (van der Hoek et al., 2005; Wu et al., 2008; Sung et al., 2010), SARS-CoV is highly pathogenic and is characterized by acute lung failure with high mortality (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003; WHO, 2004).

This contrast has generated wide interest in comparing the roles of S proteins of these two viruses in hACE2 binding and entry. The receptor-binding domain (RBD) has been mapped to amino acid residues 318–510 for SARS-CoV (Wong et al., 2004), and 476–616 for NL63 (Li et al., 2007; Lin et al., 2008). The RBDs of these two viruses bind to a largely overlapping region of hACE2 (Li et al., 2007), although the receptor-binding affinity of NL63 S is much less compared with SARS-CoV S (Lin et al., 2008; Mathewson et al., 2008). In addition, SARS-CoV S and NL63 S may cause differential downregulation of hACE2 level by induced shedding of its ectodomain from the cell surface (Haga et al., 2008; Glowacka et al., 2010). Downregulation of hACE2 is considered to be a leading cause of acute lung failure (Imai et al., 2005; Kuba et al., 2005). Finally, SARS-CoV S, but not NL63 S, is cleaved by endosomal cathepsin L, and such proteolytic cleavage is important for SARS-CoV entry (Huang et al., 2006). It should be mentioned that these studies were greatly facilitated by the utilization of lentivirus- or VSV-based pseudotype virus bearing coronaviral S proteins. These S-mediated pseudotype viruses have identical cell tropisms to the authentic coronaviruses, and their entry is also solely dependent on cellular receptors (Broer et al., 2006; Fukushi et al., 2005; Giroglou et al., 2004; Hofmann et al., 2004; Li et al., 2003, 2005b; Moore et al., 2004; Nie et al., 2004; Schwegmann-Wessels et al., 2009; Simmons et al., 2004). The use of pseudotype viruses not only provides a safe tool to analyze the entry process of highly pathogenic viruses under conventional biosafety condition, but also provides a unique system where the role of S protein in entry is not interfered by other viral proteins.

Nevertheless, compared to the tremendous efforts focused on SARS-CoV S, little attention has been given to the S protein of NL63. Here, we established an efficient pseudotype virus system for NL63 and used it to characterize the contributions of various regions of NL63 S protein in mediating plasma membrane translocation, incorporation into pseudotype virions, receptor association, and pseudotype virus entry.