Virology 393 (2009) 265–271

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

SARS-coronavirus spike S2 domain flanked by cysteine residues C822 and C833 is important for activation of membrane fusion

Ikenna G. Madu, Sandrine Belouzard, Gary R. Whittaker *

C4127 Veterinary Medical Center, Department of Microbiology and Immunology, Cornell University, Ithaca NY 14853, USA

ARTICLE INFO

Article history:
Received 8 May 2009
Returned to author for revision 27 May 2009
Accepted 30 July 2009
Available online 29 August 2009

Keywords:
SARS coronavirus
Spike protein
Membrane fusion
Disulfide loop

ABSTRACT

The S2 domain of the coronavirus spike (S) protein is known to be responsible for mediating membrane fusion. In addition to a well-recognized cleavage site at the S1–S2 boundary, a second proteolytic cleavage site has been identified in the severe acute respiratory syndrome coronavirus (SARS-CoV) S2 domain (R797). C-terminal to this S2 cleavage site is a conserved region flanked by cysteine residues C822 and C833. Here, we investigated the importance of this well conserved region for SARS-CoV S-mediated fusion activation. We show that the residues between C822–C833 are well conserved across all coronaviruses. Mutagenic analysis of SARS-CoV S, combined with cell–cell fusion and pseudotyped virion infectivity assays, showed a critical role for the core-conserved residues C822, D830, L831, and C833. Based on available predictive models, we propose that the conserved domain flanked by cysteines 822 and 833 forms a loop structure that interacts with components of the SARS-CoV S trimer to control the activation of membrane fusion.

Introduction

The severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in 2002 causing a global epidemic. The outbreak resulted in about 8000 cases with a fatality of about 10% until it was quarantined (Drosten et al., 2003; Fouchier et al., 2003). SARS-CoV still maintains a significant threat to human health, as novel viruses such as these still present a possibility for re-emergence into the human population so an understanding of the mechanics of entry is crucial in order to develop effective treatment.

Coronaviruses are enveloped viruses with positive sense RNA genomes that commonly cause respiratory and enteric diseases within a wide host range (Holmes, 2003). Entry of these viruses is mediated by the viral spike glycoprotein S and a receptor on the target cell. The viral spike glycoprotein can be cleaved into S1 and S2 domains (Bergeron et al., 2005; Du et al., 2007; Follis et al., 2006; Huang et al., 2006; Jackwood et al., 2001; Kawase et al., 2009; Watanabe et al., 2008; Yamada et al., 1998). The S1 domain of the viral spike protein dictates tropism and is responsible for mediating receptor binding (Chen et al., 1997; Han et al., 2007; Hensley and Baric, 1998; Hofmann et al., 2006; Lewicki and Gallagher, 2002; Li et al., 2003, 2007; Schultze et al., 1996; Thorp and Gallagher, 2004; Wentworth and Holmes, 2001). The S2 domain is responsible for mediating membrane fusion between the virus and host cell, with strong sequence conservation within the family (Bosch et al., 2004; Chu et al., 2006)—hence the mechanics of fusion can be expected to be conserved across the *Coronaviridae*.

Based on structural similarities, the SARS-CoV S glycoprotein is a class 1 membrane fusion protein (Schibli and Weissenhorn, 2004). The S2 domain contains two heptad repeat regions, HR1 and HR2, as well as a fusion peptide. Following conformational changes based on receptor binding or change in pH, the S2 domain drives fusion of the viral and host cell membranes to allow virus entry. Observations of cell surface expressed SARS-CoV spike protein, indicated that most of the protein was not cleaved at the S1–S2 boundary and with at best limited cleavage possible (Song et al., 2004; Xiao et al., 2003). It is generally considered that S1–S2 cleavage is not directly linked to fusion peptide exposure in the case of SARS-CoV, or any other coronavirus (Bosch and Rottier, 2008). However, it has recently been shown that SARS-CoV S can be proteolytically cleaved at a downstream position in S2, at residue 797 (Belouzard et al., 2009), and a highly conserved region C-terminal to the cleavage site has been characterized and identified as critical for fusion (Madu et al., 2009).

Downstream of these conserved core residues we observed another set of conserved residues flanked by cysteines 822 and 833. These flanks represent two of the 39 cysteines in S that are likely to form intra-disulfide bonds within S. Cysteine residues and their roles in mediating entry either in the receptor binding region or in the cytoplasmic tail have been well documented for coronaviruses (Petit et al., 2007; Petit et al., 2005; Thorp et al., 2006; Ye et al., 2004). For other families of viruses, cysteine residues have been key players for driving entry (Matthias and Hogg, 2003; Matthias et al., 2002) and fusion (Delos et al., 2008; Delos and White, 2000; Parrott et al., 2009; Rai et al., 2004). In this study, we investigated the importance of a conserved domain in SARS-CoV S2 flanked by cysteines 822 and 833 by carrying out a comprehensive mutagenesis study. Using cell–cell

* Corresponding author. Fax: +1 607 253 3385.
E-mail address: grw7@cornell.edu (G.R. Whittaker).

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.07.038

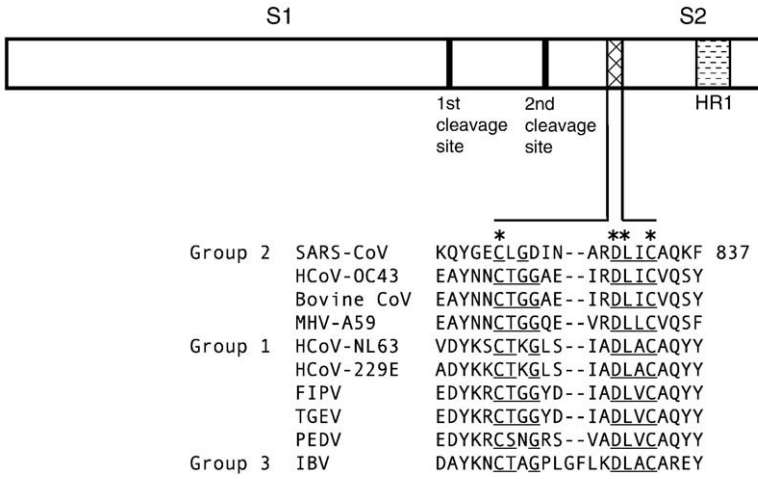

Fig. 1. Schematic and alignment of the cysteine-flanked region. A schematic representation of the coronavirus spike with hallmark domains alongside the region of interest. A multiple sequence alignment of the S protein from representative coronavirus groups was performed. Residues that are conserved directly or in terms of properties are underlined and asterisked residues represent 100% conservation within the CoV family. Virus abbreviations and Genbank accession numbers are as follows: SARS-CoV, accession no. AAP13441; HCoV-NL63, Human coronavirus NL63 Amsterdam accession no. AAS58177; HCoV-229E, Human coronavirus 229E accession no. AAG48592; HCoV-OC43, Human coronavirus OC43 ATCC VR-759 accession no. AAR01015; IBV, Infectious Bronchitis virus Beaudette strain accession no. AAY24433; MHV-A59, Mouse Hepatitis virus A59 accession no. AAB86819; PEDV, Porcine epidemic diarrhea virus LZC accession no. ABM64776; Bovine CoV, Bovine coronavirus R-AH187 accession no. ABP38295; ABP87990; FIPV, Feline infectious peritonitis virus WSU 79-1146 accession no. YP239355; TGEV, Transmissible gastroenteritis virus Purdue PUR46-MAD accession no. NP058424.

fusion and pseudovirus assays, we show that this domain is critical for the activation of SARS-CoV S-mediated membrane fusion and virus entry.

Results

Bioinformatic analysis of the SARS-CoV S2 domain flanked by cysteine residues C822 and C833

A common feature of regions within a viral glycoprotein that are required for entry is that they show a high degree of conservation within a virus family. We therefore performed a multiple sequence alignment of the spike protein of representative coronaviruses, with a focus on the domain flanked by cysteine residues C822 and C833. This bioinformatic analysis confirmed a high degree of conservation in the region (Fig. 1). Indeed, residues C822, D830, L831, and C833 of the SARS-CoV S represent some of the most conserved residues in that region and across the *Coronaviridae*.

Cloning and expression of WT and mutant SARS-CoV S glycoproteins

To test the fusogenic properties of the conserved S2 domain flanked by cysteine residues C822 and C833 a series of mutations were introduced into a SARS-CoV S protein-expressing vector to generate the following mutants: C822S, G824A, D825A, N827A, A828S, D830L, L831D, C833S, a double cysteine mutant (C822S/C833S), a deletion of the residues between the cysteines (Δ823–832) and a Gly-Ala-Gly (GAG) loop mutant to replace the residues between the cysteines. The rationale for these mutations was as follows: residues G824, D825, N827, and A828 were simply mutated to alanine (or serine in the case of A828) to modify their chemical nature in an innocuous manner; the conserved residues C822 and C833 were mutated to serine to change the chemical nature of the cysteines and prevent normal disulphide bond formation; the C822S/C833S double mutant was designed to curb the possibility of spurious disulfide bonding occurring with unpaired cysteines; D830L and L831D mutants were generated to modify the chemistry of each original residue with the corresponding conserved residue, as both are 100% conserved; and to further address how residues within the proposed loop might be important, we generated a loop deletion mutant Δ823–832 and a flexible tripeptide GAG loop (Kwong et al., 1998) between the flanking cysteines.

In order to evaluate the effect of the mutations on the fusogenic properties of SARS-CoV S protein, we first verified the cell surface expression of the mutants. The level of surface expression of the point mutants was verified quantitatively after transfection of expression vectors bearing mutant or wild type S protein in BHK-21 cells, at both 37 °C and 32 °C, followed by biotinylation and immunoblotting. At 37 °C, with the exception of the N827A mutant, we observed a tolerable surface expression of many of the mutants (Fig. 2A), but at 32 °C we observed an overall better surface expression of all mutants expressing at >65% of wild type levels (Fig. 2B) so this temperature was used in the corresponding surface expression fusion assays as well as in the generation of pseudotyped virions.

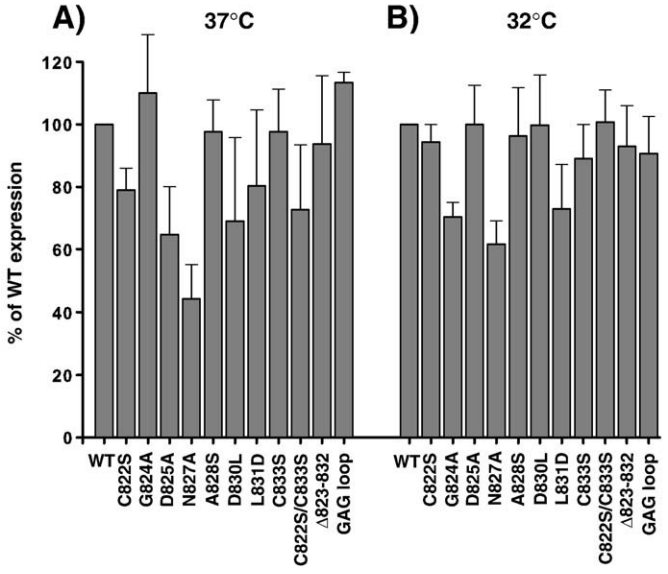

Fig. 2. Effect of point mutants on spike protein surface expression. BHK cells were transfected with plasmids encoding wild type SARS-CoV S or alanine mutants at either 37 °C (panel A) or 32 °C (panel B). The cells were labeled with Sulfo-NHS-SS-biotin and lysed. Lysates were affinity purified with NeutraAvidin beads and resolved by SDS-PAGE and Western blot using anti C9 tag antibody. The biotinylation assay was repeated three times and the results from the Western blots were quantified using IP lab software and plotted in Sigma Plot 9.0. Error bars represent standard deviation of the mean.