SITE DIRECTED MUTAGENESIS OF THE MURINE CORONAVIRUS SPIKE PROTEIN

Effects on Fusion

E. C. W. Bos, L. Heijnen, and W. J. M. Spaan

Institute of Virology, Faculty of Medicine
University of Leiden
The Netherlands

ABSTRACT

Mutations were introduced in the transmembrane region of the spike protein of the murine coronavirus A59. The maturation of these mutant S proteins was not affected, they were all expressed at the cell surface, and became acylated, however some mutant S proteins did not induce cell-to-cell fusion. An I→K change in the middle of the predicted transmembrane (TM) anchor and mutation of the first three cysteine residues of the TM domain resulted in a fusion-negative phenotype. We propose a model by which these data can be explained.

INTRODUCTION

The spike (S) protein of the murine coronaviruses has several important features. The glycoprotein has been shown to bind to the receptor on the host cell¹, and is capable of inducing cell-to-cell fusion². Furthermore the spike protein can induce neutralizing antibodies³.

The murine coronavirus Spike protein is synthesized as a 110 kDa core protein that is co-translationally glycosylated in the ER to become a 150 kDa glycoprotein. During transport from the ER to and through the Golgi stacks, the protein matures to its 180 kDa form. Subsequently, part of the molecules are cleaved into two subunits of 90 kDa (S1 and S2). In recent years several groups have shown that cleavage of S is not a prerequisite for cell-to-cell fusion. However, cleaved S induces fusion more rapidly^{4, 5, 6}.

Although it has been known for several years that the spike protein functions as a fusion protein, the fusion mechanism is still unknown. Gallagher et al.⁷ showed that fusion could be induced at low pH when mutations occurred in the first heptad repeat. Furthermore, Grosse and Siddell⁸ found that spike proteins that were resistant to neutralizing antibody

Figure 1. Schematic representation of the transmembrane anchor of the coronavirus spike protein. The amino acid sequence of the wild type protein is depicted at the upper line. For the mutants, only the changed amino acids are depicted. Charged residues at the N- and C-terminus of the TM anchor are marked +. Conserved residues (the WYV domain and the cysteine residues) are in boxes.

11F, did contain mutations in the second heptad repeat of the S2 sub-unit. This monoclonal antibody 11F had previously been shown to inhibit cell fusion, but not the virus-receptor interaction⁹. Thus, the S2 sub-unit of the S protein is very likely to be involved in the pH-related conformational changes that are required for fusion. In this study we have focused on the role of the transmembrane region in cell fusion.

RESULTS

The TM anchor is defined as the region between charged residues that is potentially inserted into the membrane. The charged residue at the C-terminus of the TM anchor functions as a stop translocation signal. From a comparison of the TM anchor of all coronavirus S genes sequenced to date, some general features can be deduced. Firstly, at the N-terminus of the TM seven large hydrophobic residues are located, which are conserved in all S proteins. Secondly, this domain is followed by 11 to 22 hydrophilic amino acids, containing several cysteines residues. The cysteine residues in this region are candidates for palmitoylation. Finally, the length of the TM domain is unusual large.

We have introduced several mutations in the TM domain as indicated in Fig.1 and analyzed the expression and the fusogenicity of the mutant S protein.

First, transport of the different mutants was studied. The proteins were expressed in L-cells, using the vaccinia T7 expression system. In a pulse-chase experiment, the endo-H profiles of the mutants was determined. All mutants were transported to the medial Golgi. Transport of the mutants to the trans Golgi stacks was not affected, since all mutants were partially cleaved into the two 90 kDa sub-units (Table 1).

By using immunofluorescence it was demonstrated that all mutants were transported to the cell surface. However, not all mutants were fusogenic. M43 was as fusogenic as the

Table 1. Characteristics of the spike mutants.

	Cleavage	Cell surface expression	Acylation	Cell-to-cell fusion
wt	+	+	+	+
m40	+	+	+	–
m41	+	+	+	–
m42	+	+	+	+
m43	+	+	+	+

Figure 2. Possible models of the spike protein of MHV before (A) and after conformational changes have occurred (B and C). The S1 and S2 sub-units of the monomeric form of the spike protein are indicated. The highly conserved WYV domain is depicted as a box. The charged residue that functions as transfer stop signal is indicated by + at the cytoplasmic side of the membrane.

wild type spike protein, the fusion induced by m42 was delayed and the syncytia remained smaller, and m40 and m41 did not induce syncytia (Table 1).

For influenza virus, it has been shown that the degree of palmytoilation of the fusion protein can affect fusogenicity. The HA protein of some influenza strains is not fusogenic when palmytoilation is blocked^{10, 11}. For other strains, however, block of acylation had no effect on fusion^{12, 13}. To study the effect of the cysteine mutations on acylation of the murine coronavirus S protein, L-cells were labelled with ³H-palmitic acid, and subsequently subjected to immunoprecipitation. Unfortunately all mutants became acylated, albeit to a different extent. Mutants m41 and m42 were less acylated, suggesting that some of the cysteine residues that were changed in these mutants are involved in acylation (Table 1).

We propose a model, by which our data can be explained, Fig.2. We hypothesize that after binding of S to the receptor, some conformational changes in both S1 and S2 occur as indicated in Figure 2. Either, the entire protein is pulled out of the membrane, thus exposing the highly conserved WYV domain (Fig.2B). Or as depicted in Figure 2C, the TM region of S could be twisted in the membrane, such that it becomes inserted in a different angle. Both proposed conformational changes require a large TM domain. Due to acylation the cysteine residues become hydrophobic, and can therefore be inserted into the lipid bilayer. The proposed changes in the S2 domain are not possible for our mutants m40 and m41. The charged residue in m40 may serve as a stop-transfer signal during translocation of the S protein across the ER membrane. As a result the TM domain of m40 is fixed in the membrane. In the absence of acylation of the cysteine residues which have been mutated in m41 the hydrophobic feature of the cysteine rich domain is less pronounced resulting in again a fixed

TM domain The phenotype of our mutants indicates that both the length of the TM and a position effect of acylated cysteines might be involved in fusion

Finally, we have developed an assay by which we can test the effect of mutations in S or other structural proteins on infectivity To this end, we transfected vaccinia virus infected L cells with four different constructs encoding for S, M, N and sM As RNA, the DI-RNA¹⁴ was also introduced in the cells The release of DI RNA containing particles was analyzed by adding helpervirus MHV A59 to the medium and infecting a new monolayer of L-cells using this mixture Replication of the DI-RNA was analyzed following several undiluted passages

It was demonstrated that DI-RNA was only transferred to helpervirus-infected L-cells when DI-RNA was co-expressed with all structural proteins In the absence of the structural proteins no DI-RNA was observed following undiluted passage Although not proven directly, we assume that the DI-RNA which was released into the medium of the transfected cells was packaged into a virus like particle

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