Murine Coronavirus Spike Glycoprotein

Receptor binding and membrane fusion activities

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1. INTRODUCTION

The mature coronavirus spike is a large, oligomeric, type I integral membrane glycoprotein that projects about 20 nm from the surface of infected cells and virions. In this extracellular position, the spikes function to bind the cellular receptors extending from opposing membranes. Following receptor binding, largely hypothetical structural changes take place to generate spike conformations that are capable of mediating fusion of the juxtaposed membranes. Membrane fusion creates pores for entrance of genomes into uninfected cells.

In their role as agents of viral genome entry and dissemination, the spikes encounter varied physical, chemical and biological conditions. These different environmental conditions can impact spike protein structure, often impairing their participation in the infection process. For example, spikes may complex with antibodies (Dalziel *et al.*, 1986), or spikes may become exposed to changes in pH and temperature (Sturman *et al.*, 1990), in ways that can render them incompetent during virus entry. To persist in these changing environments, coronavirus populations adapt by natural selection and this process fixes mutations into the spike genes. With extended selection in different niches, spikes collectively establish a rich genetic diversity that can be exploited by coronavirologists. For example, researchers interested in the tropism and pathogenesis of the coronaviruses have discovered that naturally-occurring spike mutations can lead to

The Nidoviruses (Coronaviruses and Arteriviruses).
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dramatic changes in the targeting of *in vivo* infection (Leparc-Goffart *et al.*, 1998, Sanchez *et al.*, 1999).

This chapter describes results extending from investigations on the entry of a unique strain of murine coronavirus, the JHM strain of murine hepatitis virus (MHV) (Cheever *et al.*, 1949). MHV strain JHM is extremely pathogenic to mice, causing acute, fatal encephalitis. In tissue culture, infectious JHM viruses are difficult to obtain in abundance because the spikes are unstable at 37°C temperature and slightly basic pH. Amplification of JHM in tissue culture naturally selects for variants of greater stability. These variants contain mutations in spike genes that correlate with reduced *in vivo* pathogenicity (Fazakerley *et al.*, 1992) and reduced spike-mediated membrane fusion activity (Gallagher *et al.*, 1990). JHM and its tissue-culture variants provide the source material to study the molecular basis of coronavirus entry, dissemination and pathogenesis.

2. METHODS AND RESULTS

Spike protein synthesis begins with the co-translational insertion of nascent chains into the lumen of the endoplasmic reticulum (ER). Newly synthesized proteins assemble into oligomers, probably dimers (Vennema *et al.*, 1990) and then proceed through the exocytic pathway of the infected cell. Spikes are heavily glycosylated and are palmitoylated near their carboxy-terminal transmembrane anchors. The most notable additional spike modification takes place within the trans-Golgi network, where cellular endoprotease(s) recognize a central stretch of multibasic residues found within spikes of a selected group of strains (Cavanagh, 1995). This proteolysis generates an amino-terminal, peripheral S1 of 769 amino acids, and carboxy-terminal, integral-membrane S2 of 607 amino acids (Fig. 1). These two fragments remain noncovalently associated for variable time periods, depending on the strain under investigation.

During a natural coronavirus infection, most spikes that leave the ER associate with other viral components to become incorporated into secreted virions (Opstelten *et al.*, 1995). To avoid this loss of spike proteins from cells via their incorporation into secreted virions, most of our studies involve spike biosynthesis in the absence of other coronavirus proteins, a process that is accomplished by expressing spike cDNA from vaccinia vectors (Gallagher, 1997). We synthesize many different spike mutants ("strains") in this way, and the spikes are routinely investigated using a series of

straightforward assays. First, spikes are monitored for synthesis and post-translational transport through the exocytic pathway by pulse-chase radiolabelling with [^{35}S] methionine, immunoprecipitation and autoradiographic detection after electrophoresis. Second, spikes inside cells or on plasma membranes are assayed for their ability to bind receptors by measuring their adsorption to a soluble form of the murine hepatitis virus receptor (sMHVR). Third, spikes on plasma membranes are further assayed for their ability to mediate membrane fusion by measuring syncytium formation following incubation with suitable target cells. We have used these three approaches to identify structure-function relationships in the coronavirus spike.

2.1 Biosynthesis of spike proteins: Formation of the MHVR-binding site

One question about coronavirus entry concerns the binding sites for MHVR on the spike proteins. Binding of MHVR to spikes is thought to promote the formation of spike conformations capable of membrane fusion. However, the way that this hypothetical induction might occur, or even how the MHVR interacts with spikes, is largely unknown. Our studies of binding between soluble MHVR proteins and spikes are aimed at addressing these questions.

The sMHVR-Fc (Fig. 1) is a bifunctional binding agent. Its amino-terminal region is identical to the "N" domain of mouse CEACAM1a (Beauchemin *et al.*, 1999) and will therefore bind MHV spike proteins (Dveksler *et al.*, 1993), while its carboxy-terminal region is a human IgG1 Fc and will therefore bind to protein A or G in immunoprecipitations. Using sMHVR-Fc in immunoprecipitations of metabolically labeled spikes, we discovered that spikes synthesized within a 10 min pulse period were not efficiently captured. Chase periods of 1 h or more were required for spikes to develop structures capable of recognition by sMHVR-Fc (see, for example, Fig. 4). Newly-synthesized spikes were sedimented down sucrose gradients, and the spikes in gradient fractions were captured by either sMHVR-Fc or by polyclonal anti-spike antiserum. The results revealed that the antiserum captured a spectrum of spike conformations ranging from ~ 5S to ~16S, while the sMHVR-Fc only captured the small amount of ~15S material that is consistent with the sedimentation rate of spike dimers (Fig. 2). Thus the formation of the sMHVR-Ig binding site was correlated with formation of spike protein oligomers.

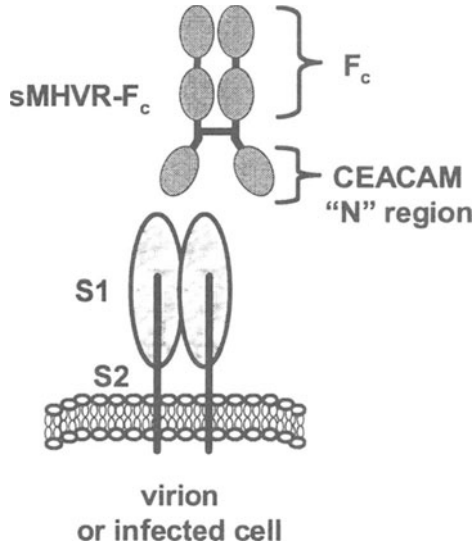

Figure 1. Depiction of spike and sMHVR-Fc proteins. The S1 subunit is attached to S2 through noncovalent bonding. The "N" region of CEACAM binds directly to S1. sMHVR-Fc is a disulfide-linked homodimer.

The results of Kubo *et al.* (1994) indicate that cDNAs encoding only the amino-terminal 330 residues of spike could be expressed from vaccinia vectors, and the secreted peptide fragments could bind to immobilized MHVR proteins. If the MHVR-binding site was dependent on spike oligomerization, then the S_{330} fragment should harbor oligomerization determinant(s). We tested this possiblity by expressing two cDNAs within a single cell culture; one encoded S_{330} and the other encoded a complete S1 fragment, S_{769}. The existence of S_{769}:S_{330} hetero-oligomers would provide support for the hypothesis. Indeed, a monoclonal antibody (mAb J.2.6; Fleming *et al.*, 1983) with specificity for residues between 510-540 (data not shown), did indeed immunoprecipitate both S_{769} and S_{330} from the culture lysate (Fig. 3; lane 4). The same mAb captured only S_{769} when produced alone (lane 2), and failed to recognize S_{330} when produced alone (lane 3). This data supported the contention that an oligomerization region lies within the amino-terminal 330 residues of spike.

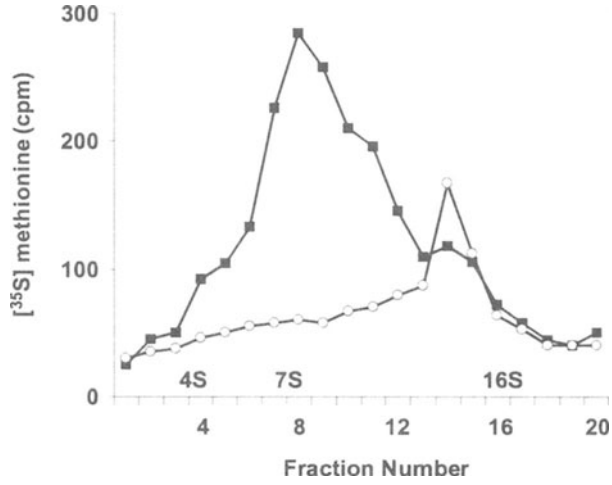

Figure 2. Capture of newly-synthesized [^{35}S] spikes after their sedimentation on sucrose gradients. HeLa cells were infected with recombinant vaccinia vectors encoding spike proteins. After pulse-labelling with [^{35}S] methionine from 7.5 to 8.0 hours postinfection, cells were dissolved with nonionic detergent and lysates were sedimented on sucrose gradients. Spikes in gradient fractions were immunoprecipitated with either anti-JHM antiserum (solid squares) or with sMHVR-Fc (open circles) and the captured radioactivity was counted by scintillation spectrometry. Markers for sedimentation rate were horseradish peroxidase (4S), immunoglobulin G (7S) and β-galactosidase (16S).

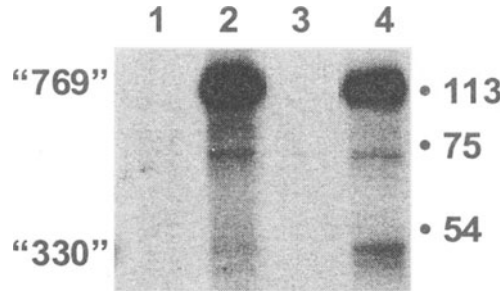

Figure 3. Co-immunoprecipitation of S_{769} and S_{330} fragments by mAb J.2.6. Spike fragments were synthesized in HeLa cells from vaccinia vectors and were metabolically radiolabelled with [^{35}S] methionine. After immunoprecipitation of proteins from lysates, SDS-polyacrylamide gel electrophoresis and flourography were performed to reveal the "769" and "330" fragments. The position of molecular weight markers is indicated at the right in kilodaltons. Lane 1: No S (negative control). Lane 2: S_{769}. Lane 3: S_{330}. Lane 4: S_{769} + S_{330}.

3.2 Cell-surface presentation of spike proteins: Stability of the S1-S2 interaction and its relationship to the membrane fusion reaction

Oligomerized S1-S2 complexes encoded by JHM are exceedingly thermolabile. At 37°C, the infectivity of JHM virus particles declines rapidly as spikes become denatured. Tissue-culture growth of JHM gives rise to more thermostable variants, some of which have fixed mutations into the spike gene. Some of these thermostability mutations are easy to identify as they are relatively large ~ 450 nucleotide, in-frame deletions within the S1-encoding region (Gallagher *et al.*, 1990). The "deletion-prone region" (DPR) of S1 is always within residues ~ 420 to ~ 600.

Mutations associated with increased thermostability are also associated with long-lived S1-S2 interactions. This was demonstrated by comparing the kinetics of S1-S2 formation and decay for S_{JHM} and one of its variants, $S_{\Delta DPR2}$ (Δ437-585). Pulse-chase radiolabelling, immunoprecipitation with sMHVR-Fc, and S1 / S2 detection after electrophoresis revealed that a stable, cell-associated S1-S2 complex was never observed for S_{JHM} (Fig. 4A). It appeared that an S1-S2 complex decayed quickly after it was formed. S1 was always found in culture media, while free S2 could not be identified in cells or media and was presumably rapidly degraded. In contrast, $S1_{\Delta DPR2}$ -S2 complexes were readily captured from cell lysates and relatively little free $S1_{\Delta DPR2}$ was found in culture media (Fig. 4B). These results depicting stable maintenance of $S1_{\Delta DPR2}$ on the plasma membrane were corroborated by our finding that cultures synthesizing $S_{\Delta DPR2}$ readily absorbed exogenously-added sMHVR-Ig at levels over 10 times that of S_{JHM} (data not shown). Thus the JHM strain of MHV can accumulate spike mutations that increase the duration of the S1-S2 association and thereby help to maintain virus infectivity at 37°C.

The JHM spike protein is far more potent at mediating membrane fusion than its more thermostable ΔDPR variants. This was demonstrated by performing a series of assays for membrane fusion-dependent cytoplasmic mixing between spike-bearing (effector) and MHVR-bearing (target) cells. To measure the extent of fusion-dependent cytoplasmic mixing, we used the assay of Nussbaum *et al.* (1994) which involves infecting target cells with vCB21R-lacZ (Alkhatib *et al.*, 1996), thereby introducing a transcriptionally-silent β galactosidase reporter gene under T7 promoter control. Since spike-bearing effector cells are infected with vTF7.3 (Fuerst *et al.*, 1987), which encodes T7 RNA polymerase, effector:target cell fusion brings T7 RNA polymerase in contact with the $T7_{pro}$-lacZ and β-galactosidase is synthesized.

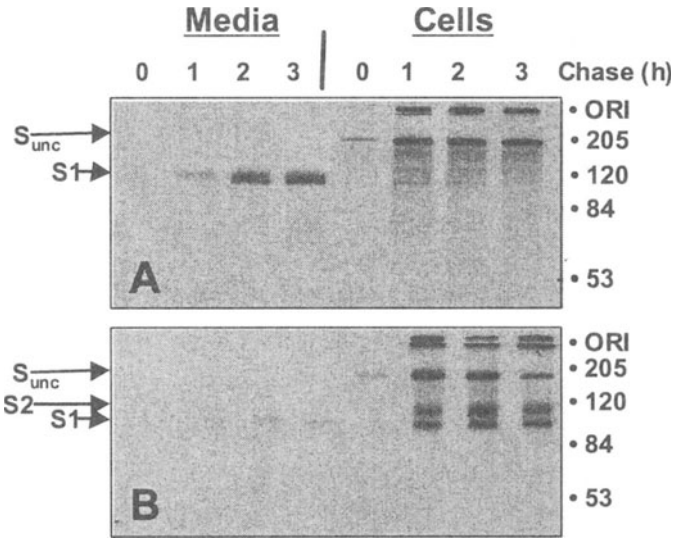

Figure 4. Evaluation of spike protein synthesis, proteolytic fragmentation and elution into media. HeLa cells producing spike proteins were radiolabelled with [^{35}S] methionine for 10 min and then chased for the indicated time periods before cell lysis and immunoprecipitation with sMHVR-Fc. Captured spike proteins were revealed after SDS-polyacrylamide gel electrophoresis and flourography. Molecular weight markers are indicated at the right in kilodaltons. S_{unc} denotes the uncleaved precursor of S1 and S2 fragments. Panel A: S_{JHM}. Panel B: $S_{\Delta DPR2}$. This figure was adapted from Krueger *et al.*, submitted.

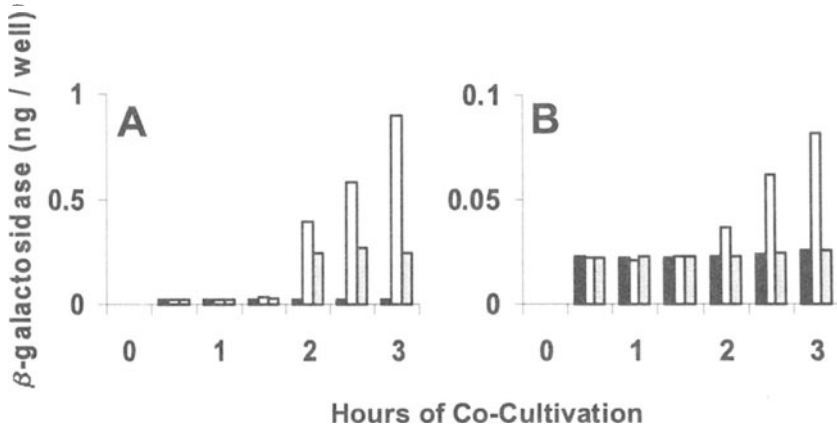

Figure 5. Quantitative assessment of intercellular fusion indicating that S_{JHM} is uniquely capable of MHV receptor-independent syncytium formation. Cytoplasmic mixing that occurs within syncytia of co-cultivated cells was measured by β-galactosidase production, as described in the text. Solid bars; no spikes on effector cells (negative control). Open bars; S_{JHM} effector cells. Shaded bars; $S_{\Delta DPR2}$ effector cells. Panel A: Hela-MHVR (+R) target cells were overlaid onto the designated S-bearing effector cell monolayers, and lysate-associated β-galactosidase activities were measured at the indicated times after co-cultivation. Panel B: HeLa (-R) target cells were used. This figure was adapted from Krueger *et al.*, submitted.

When target cells displayed the MHVR (Rao and Gallagher, 1998), the $S_{\Delta DPR2}$ stimulated β-galactosidase production that was about 25% the level of S_{JHM} (Fig. 5A). A more striking discovery was made from a parallel fusion assay in which target cells lacked the MHVR. Here the $S_{\Delta DPR2}$ did not stimulate any β-galactosidase production, indicating that the MHVR is required to generate a fusion-competent conformation. However, S_{JHM} could indeed fuse with cells lacking the MHVR, as assessed by β-galactosidase induction (Fig. 5B). A hyperactive membrane fusion activity that can occur even without prior binding of the MHVR is therefore correlated with the extremely labile $S1_{JHM}$-S2 interaction.

4. DISCUSSION

The JHM spike is a very large glycoprotein of 1376 amino acids and 20 predicted asparagine-linked carbohydrates. Its folding in the ER is complex, likely requiring assistance from numerous ER chaperones. Using sMHVR-Fc as an oligomer-specific immunoadhesin, we found that full-length spikes are synthesized and then slowly folded into oligomers in a process requiring about one hour. It is interesting that spike proteins were not captured by soluble MHV receptors until they formed oligomers (Fig. 2), indicating that additional folding events take place during or shortly after oligomerization to form the MHVR-binding site.

The large spike protein can be functionally subdivided in that the MHVR-binding site can form when only about one-fourth of the protein (residues 1-330) are synthesized (Kubo *et al.*, 1994). We found that the S_{330} fragment contains region(s) conferring homo-oligomerization (Fig. 3). It is not yet clear whether each monomer of the spike homo-oligomer contains a separate and independent MHVR-binding site. It is also not clear whether the binding of MHVR to this amino-terminal region causes conformational changes in the carboxy-terminal S2 fragments that are thought to carry out the membrane fusion reaction. Such changes would have to occur however if the MHVR is to be considered a trigger for spike-mediated membrane fusion.

Limiting step(s) on the pathway to S1-S2 complexes on the plasma membrane occurs between formation of the uncleaved and cleaved spike oligomer. This is evident from the detection of uncleaved spikes throughout every 3 h chase period (see Fig. 4). However, small proportions of each spike population do progress through the exocytic pathway during the chase periods. For JHM, the spikes that reach the cell surface do not maintain their MHVR-binding sites. The S1 fragment, along with its site for receptor interaction, is rapidly jettisoned. The S2 that remains cell-associated is

rapidly degraded. Tissue-culture adaptation selects for mutations that delay this process of S1-S2 elution. One example of this is depicted in this chapter – the deletion of S1 residues 437-585 (ΔDPR2) permits stable S1-S2 complexes (Fig. 4). Increased stability of the S1-S2 complex was correlated with reduced membrane fusion activity, as the $S_{\Delta DPR2}$ was far less potent in mediating intercellular fusion despite its continued accumulation on cell surfaces (Fig. 5).

It is clear that the coronaviruses are capable of encoding a spectrum of different spike structures. Some of these structures may be extremely unstable, thereby creating a relatively small free energy barrier between the so-called "native" and "fusion-active" conformations. The JHM spike is the prototypic example here. For JHM, the one-way conversion to the fusion-active conformation does not even require the free energy that is presumably released upon MHVR interaction, making it capable of "MHVR-independent" development of syncytia. Maintenance of virus infectivity at 37°C requires increased stability of the protruding spikes, and this occurs through mutation and selection. Increased stability of the native conformation necessarily places a higher energy barrier in front of the "fusion-active" state, and thus the presumed energy of MHVR interaction is required to activate membrane fusion by mutant spikes. Relative to the prototype JHM, viruses with stabilized spike proteins are associated with reduced *in vivo* virulence and with slower spread of infection (Fazakerley *et al.*, 1992). Perhaps this restricted dissemination is explained by *in vivo* environments that are unable to drive the tissue-culture adapted spikes through a high-energy barrier and into the fusion-active state.

ACKNOWLEDGMENTS

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