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**Coronavirus Spike Proteins in Viral Entry and Pathogenesis**

Thomas M. Gallagher* and Michael J. Buchmeier†

*Department of Microbiology and Immunology, Loyola University Medical Center, Maywood, Illinois 60153; and †Division of Virology, Department of Neuropharmacology, Scripps Research Institute, La Jolla, California 92037

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Coronaviruses comprise a large and diverse family of enveloped, positive-stranded RNA viruses. The Coronaviridae exhibit broad host range, infecting many mammalian and avian species and causing upper respiratory, gastrointestinal, hepatic, and central nervous system diseases. In humans and fowl, coronaviruses primarily cause upper respiratory tract infections, while porcine and bovine coronaviruses establish enteric infections that result in severe economic loss. Coronaviruses of laboratory mice are, for historical reasons, designated as mouse hepatitis viruses (MHVs), but among these only a subset are strictly hepatotropic. Enteric strains are commonly found in rodent colonies and neurotropic strains are exploited to study central nervous system infection and demyelinating disease (Perlman et al., 2000). The extraordinary variations in host range and tissue tropism among coronaviruses are in large part attributable to variations in the spike glycoprotein. The S protein is a large, type I membrane glycoprotein that contains distinct functional domains near the amino (S1) and carboxy (S2) termini. These spikes function to define viral tropism by their receptor specificity and perhaps also by their membrane fusion activity during virus entry into cells. Recently their natural variation has attracted the attention of researchers interested in determinants of viral host range, virus entry, and virus–receptor interactions and their relationship to tropism.

Evidence supporting a role for spike protein projections as agents of organ tropism and pathogenesis began with comparative studies of different naturally occurring MHV strains. In essence, nucleotide sequencing revealed that alterations in virus virulence were most closely associated with differences in the spike gene. Thus, it is likely that both the receptor recognition and membrane fusion properties must be investigated for a complete view of coronavirus pathogenesis.

The challenge now is to understand, in mechanistic terms, how mutations in spike proteins alter in vivo virulence. This challenge is difficult in the absence of structural data for any S protein. What is known is that the peripheral S1 portion can independently bind cellular receptors while the integral membrane S2 portion is required to mediate fusion of viral and cellular membranes (Fig. 1). While natural genetic variability is most extreme in the S1 fragment, S2 changes are also found in mutants with novel in vivo infection characteristics. Thus, it is likely that both the receptor recognition and membrane fusion properties must be investigated for a complete view of coronavirus pathogenesis.

The distribution of coronavirus receptors is critical to the pathogenic outcome. In this regard, it is notable that coronavirus spikes exhibit a range of receptor specificities; MHVs enter after binding members of a pleiotropic family of carcinoembryonic antigen–cell adhesion molecules (CEACAMs); feline and porcine coronaviruses bind metalloproteases; and bovine coronaviruses recognize 9-O-acetylated sialic acids (Holmes and Dveksler, 1994). Without detailed structural data for spikes or these receptors, insights into this initial entry stage have relied largely on identifying the minimal spike and receptor peptide fragments required for binding. These studies are relatively advanced for the MHVs, where it is known that an amino-terminal fragment of 330 residues (about one-fourth of the spike ectodomain) encompasses the receptor-binding site (Kubo et al., 1994). Conversely, the amino-terminal, immunoglobulin-like "N" domain of mu-
The precise affinities of the different spike–receptor interactions have not yet been determined. For many reasons, affinity information may be crucial to understanding mechanisms of coronavirus pathogenesis and evolution. The receptors for the MHVs are part of the large CEACAM gene family, and family members are known to differ in their tissue distribution and in strength of binding to spike proteins (Rao et al., 1997). Moreover, CEACAM genes of differing affinities may be expressed differentially in tissues at distinct developmental stages, thereby providing the potential for focal and temporal infections. The presence of multiple receptors, each with a unique “N” domain architecture, likely also contributes to MHV evolution. In this regard it is notable that persistently infected tissue culture cells can shed MHV (strain A59) variants with an expanded tropism for human, rat, hamster, feline, and monkey cells (Baric et al., 1997). The inference is that variants had evolved that could bind efficiently with the CEACAMs produced by these species.

Affinity data may also be essential for understanding the process of virus penetration, as the free energy released from spike–receptor binding may be required to trigger the next stage in virus entry, spike-mediated membrane fusion. Those receptors with the highest binding affinity may drive the fusion reaction most effectively. This underscores the importance of describing the fusion reaction in terms of the protein conformational changes within spike–receptor complexes. Descriptions of this sort are emerging for spikes complexed with soluble CEACAM 1 receptors, which develop an instability at 37°C that is recognized by the separation of the peripheral, receptor-binding S1 fragment from the integral membrane S2 fragment (Gallagher, 1997; see Fig. 1). The S2 fragment contains a putative internal fusion peptide whose precise location is not yet defined (Luo and Weiss, 1998). S2 also contains three stretches of “amphipathic heptad repeat” sequence, the middle stretch being 120 residues long, and each of these regions has a predicted propensity to engage in coiled-coil formation (Singh et al., 1999). Thus, a conservative view, one that is consistent with current paradigms for protein-mediated membrane fusion (Skehel and Wiley, 1998), is that the energy of receptor binding permits exposure of the S2 fusion peptide such that it can intercalate into an opposing target membrane. The collapse of heptad-repeat regions into coiled-coils then brings the fusion peptide back toward the base of S2, and in the process the target (cellular) and viral membranes are brought into proximity sufficient for membrane coalescence.

While receptors may serve as inducers of the membrane fusion reaction, the unusual behavior of spikes from MHV strain JHM suggests that alternative fusion triggers also exist. JHM spikes can mediate cell–cell membrane fusion with target membranes lacking murine CEACAM receptors. This murine CEACAM-independent fusion was recently found to require exposure to slightly elevated pH values of 7.5 to 8.0 (Krueger et al., 2000). This finding, combined with the fact that pH elevation from 6.0 to 8.0 causes separation of S1 and S2 fragments (Sturman et al., 1990), led us to a view in which JHM spikes are maintained as stable S1–S2 complexes in the acidic Golgi lumen. However, once displayed on the plasma membrane, the spikes encounter elevated pH and decay rapidly into soluble S1 and integral membrane S2 fragments, and some mediate murine CEACAM-independent membrane fusion in the process.

When the unstable JHM virus is propagated extensively in tissue culture, many of the progeny viruses harbor mutations in the spike gene. Sequencing efforts in a number of laboratories have now revealed a pattern of mutations that become fixed into the JHM genome after in vitro passage. There are two fundamental changes: (1) S1 deletions that remove sequences between the receptor-binding region and the fusion-inducing fragment and (2) S2 substitution mutations that alter heptad-repeat sequences (Fig. 1). Notably, particular S2 codon changes have been independently observed—for example, L1114 within the middle heptad (Fig. 1) is a hot-spot for mutation (Gallagher et al., 1991; Wang et al., 1992; Saeki et al., 1997). Spikes with S1 deletions or S2 substitutions are unable to mediate murine CEACAM-independent fusion, and relative to JHM, they exhibit enhanced S1–S2 stability (Krueger et al., 2000). Thus, we suggest that fusion activation is related in part to the stability of S1–S2 heteromers and that mutations fixed into JHM spike genes during growth in tissue culture.

![Schematic depiction of the murine coronavirus (strain JHM) spike protein. Spikes are synthesized in the endoplasmic reticulum and posttranslationally cleaved into peripheral S1 (upper bar, aa 15–769) and integral membrane S2 (lower bar, aa 770–1376) upon transport to the cell surface. The durability of noncovalent S1–S2 interaction is altered by mutations in different regions of the spike gene; this suggests multiple S1–S2 interacting sites (dotted lines). Binding of CEACAM receptors to S1 disrupts some or all of these noncovalent interactions. This is hypothesized to expose an internal membrane fusion peptide within S2 for insertion into target cell membranes. Target cell and virion membranes may then pinch together (fuse) by the collapse of the three predicted S2 helical regions (hatched bars) into coiled-coil structures.](image)
give rise to stabilized proteins that cannot straddle the energy barrier between "native" and "fusion-active" conformations without prior murine CEACAM binding.

The unstable JHM virus is set apart from its tissue culture-adapted variants in its ability to cause a rapid, disseminated, and lethal panencephalitis (Fazakerley et al., 1992; Pearce et al., 1994; see Fig. 2). Rapid coronavirus spread in the CNS may depend on spikes that can convert into the fusion-active conformation even without induction by receptor binding. After all, the prototype receptor CEACAM 1 is barely detectable in the murine CNS (Godfraind et al., 1997). On the other hand, spike protein instability is a disadvantage in tissue culture, and stabilized variant spikes are selected. Attenuation of these variants in the mouse might be explained by failure of the in vivo CNS environment to support their conversion into fusion-active forms. These investigations relating the function of "JHM-type" spikes to CNS infection provide but one example of how diversity among coronaviruses and their receptors provides models for understanding early events in viral pathogenesis.

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