Role of Type II Transmembrane Serine Proteases in Coronavirus Production

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ROLE OF TYPE II TRANSMEMBRANE
SERINE PROTEASES
IN CORONAVIRUS PRODUCTION

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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ABSTRACT

Proteolysis of Coronavirus spike proteins is required for virus infectivity. Cleavage of spike at the appropriate time and location results in efficient activation of viral entry into host cell. Type II transmembrane serine proteases (TTSPs), specifically, transmembrane protease serine 2 (TMPRSS2) when expressed on target cells, enhance SARS coronavirus entry by activating cleavage of spike. TMPRSS2, when expressed in virus producing cells, is known to proteolytically activate influenza virus HA0 and Human Metapneumovirus F0 glycoproteins. In this study, I investigated the effect of TTSPs expressed in Coronavirus producer cells. Murine Hepatitis Virus strain A59 (MHV A59) viruses produced in the absence of TMPRSS2 were found to require the activity of target cell proteases - either cell surface serine proteases or endosomal acidophilic proteases - for entry. MHV A59 viruses produced in the presence of TMPRSS2 were less infectious overall, but a portion of the viruses contained “pre-primed” S proteins and were activated for entry in the absence of target cell protease activity. These findings indicate that coronaviruses can be proteolytically activated either in virus-producing cells or target cells and the timing of spike cleavage is crucial to virus infectivity.
CHAPTER ONE
INTRODUCTION

Viruses are obligate intracellular parasites that invade cells and subsequently subvert the host cell machinery to complete their life cycle. Entry into host cells is mediated by viral surface proteins which engage with an attachment factor or virus receptor on the target cell surface. In the case of enveloped viruses, entry is mediated by surface glycoproteins [1]. Following receptor binding, enveloped viruses enter the host cell by membrane fusion, which occurs either at the plasma membrane or after endocytosis at endosomal membranes [2-4].

Coronaviruses are enveloped, positive-sense, single-stranded RNA viruses that have a broad range of tropism, infecting mammals and birds, causing respiratory, gastrointestinal, hepatic, kidney and brain infections. Mouse hepatitis viruses (MHVs) such as MHV strains A59 and 2, have been studied extensively as models for coronavirus biology and are known to cause hepatitis, diarrhea, respiratory infections and demyelinating infections of the central nervous system[5, 6]. Human coronaviruses (H-CoVs), namely HCoV-NL-63, HCoV-229E and HCoV-OC43, cause primarily mild respiratory tract infections, while human SARS-CoV is notable as the most pathogenic, causing Severe Acute Respiratory Syndrome (SARS) [7].

Spike (S) proteins mediate CoV entry into host cells via receptor binding and membrane fusion. S glycoproteins are trimeric projections on the virus envelope and are
the sole viral mediators of attachment and entry, and hence are central determinants of virulence and host tropism[8]. S proteins are synthesized at the endoplasmic reticulum and undergo oligomerization and carbohydrate processing during maturation through the cellular secretory pathway to emerge as a heavily N-glycosylated type I membrane proteins, assembled as trimers on the virion envelope[9]. S proteins belong to the class I fusion proteins, which are synthesized as precursors requiring proteolytic cleavage by host proteases to be activated for cell entry [1, 10, 11]. For some CoVs, such as MHV A59, S cleavage can occur during maturation in virus producer cells [12], while other CoVs, such as SARS-CoV and MHV2, are produced with uncleaved spikes[9, 13-15] that have to be subsequently cleaved during entry into target cells after receptor binding [16, 17].

Proteolytic cleavage positions the S proteins in an energy-rich metastable configuration, and renders them capable, upon further refolding events, of driving the energetically unfavorable task of drawing viral and target cell membranes in close apposition.

Figure 1 provides linear depictions of SARS CoV, MHV2 and MHV strain A59 spike proteins and highlights domains involved in cell receptor binding (RBDs) and virus-cell membrane fusion. The currently known sites of proteolytic cleavage are also shown.
Figure 1 Schematic depiction of Spike proteins of SARS CoV, MHV A59 and MHV2: SARS CoV and MHV 2 spike proteins are synthesized as uncleaved glycoproteins which require target cell proteases for activation, while MHV A59 spike is cleaved during assembly. SARS CoV S undergoes a second activating cleavage at S2\(^\text{R797}\), immediately N-terminal to its fusion peptide [61, 62]. The S1 subunit contains a signal peptide (SP), the receptor binding domain (RBD) while the transmembrane S2 subunit contains the fusion peptide (FP), heptad repeat (HR) sequences HR1 and HR2 which mediate membrane fusion, a transmembrane domain (TM) and a short cytoplasmic tail (CT).

The N-terminal subunit S1 contains a receptor binding domain (RBD). The C-terminal S2 subunit contains a hydrophobic fusion peptide (FP), two heptad repeat sequences (HR1 and HR2), a transmembrane domain(TM) and a cytoplasmic tail (CT). The S1 subunit mediates receptor attachment via interactions between its RBD domain and a cellular receptor, and the S2 subunit is responsible for membrane fusion. In the case of MHV, the cellular receptor is carcinoembryonic antigen-related cell adhesion molecule (CEACAM) [18-20] and for SARS-CoV, the receptor is angiotensin-converting enzyme 2 (ACE2)[21, 22]. For both viruses, binding to the receptor triggers S protein
conformational changes that render the S2 subunit fusogenic [23-26]. Figure 2 is a model depicting MHV spike-mediated membrane fusion; binding of spike to its receptor CEACAM results in the dissociation of S1 from S2 and exposure of the fusion peptide, which is inserted into the target membrane. After the fusion peptide is anchored into the target cell membrane, the S2 subunit folds back on itself resulting in the formation of a six-helix bundle (6HB). This 6HB consists of a central coiled-coil formed by three HR1 fragments surrounded by three anti-parallel HR2 helices. The formation of the 6HB brings the cell membrane-anchored fusion peptide close to the S2 transmembrane domain, thus bringing cellular and viral membrane close to each other for membrane fusion [12, 27-30].

Figure 2. Model for coronavirus spike protein-mediated membrane fusion: (From “Role of Spike Protein Endodomains in Regulating Coronavirus Entry” Shulla, A., et al., JBC, 2009[31]) S1 subunit of spike binds to cellular CEACAM receptor and dissociates from S2. After the fusion peptide (FP) is inserted into the target cell membrane, S2 folds on itself, allowing Heptad Repeat 2 (HR2) to bind with Heptad Repeat 1 (HR1) to form a six-helix bundle (6-HB). The formation of the 6-HB brings the viral and cellular membranes in contact, facilitating membrane fusion.
There are a number of host cell proteases that cleave CoV S proteins. Furin-like proteases cleave the MHV A59 spike late in its assembly stage [32], while trypsin and endosomal proteases Cathepsin B and Cathepsin L are necessary for MHV2 S-mediated cell entry[33]. The host cell proteases that have been implicated in mediating SARS CoV entry include Cathepsin L, trypsin, elastase, plasmin, thermolysin and factor Xa[17, 34, 35]. Recent studies have also identified proteases belonging to the family of type II transmembrane serine proteases (TTSPs) as SARS CoV entry co-factors [36-38]. In all cases, the proteolytic events are thought to relieve structural constraints such that S proteins can undergo the dramatic conformational transitions needed to catalyze membrane fusions.

TTSPs are a family of membrane anchored proteases with a broad range of tissue distribution and function. Though relatively recently identified, the TTSP family members have been observed to play diverse roles in maintaining cellular homeostasis in the gastrointestinal, cardiovascular and respiratory systems[39, 40]. TTSPs that are expressed in the respiratory tract, such as TMPRSS11D (also known as HAT: human airway trypsin-like protease), TMPRSS4, TMPRSS11A, and TMPRSS2, have been shown to activate and support multicycle replication of human influenza viruses [36, 41-43]. TMPRSS2 can also activate and support replication of human metapneumoviruses (HMPV), which, like influenza viruses, are etiological agents of infectious respiratory tract diseases[44]. The enhancement of both influenza virus and HMPV infections by TMPRSS2 is due to the proteolytic activation of newly synthesized, uncleaved hemagglutinin (HA0) and F₀ proteins, the surface glycoproteins of influenza and HMPV.
viruses respectively[41, 44]. In light of the activating effects that TMPRSS2 exhibits
during SARS CoV, this study set out to investigate the effects that TMPRSS2 might have
during CoV production.
CHAPTER TWO

METHODS

**Cells:** 293T [45], 293-ACE2 (293T cells stably expressing SARS corona virus receptor angiotensin converting enzyme 2, ACE2) and HeLa-CEACAM (HeLa cells stably expressing carcinoembryonic antigen cell adhesion molecule, CEACAM [46]) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.01M sodium HEPES (pH7.4). Serum Free Media (SFM) used for plaque assay titration contained 2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.01M sodium HEPES (pH7.4). Murine 17cl1 fibroblasts [47] were grown in DMEM supplemented with 5% tryptose phosphate broth, 5% fetal calf serum and 0.01M sodium HEPES (pH7.4).

**Coronaviruses:** Murine coronaviruses MHV2 and recombinant MHV A59 viruses used in this study were propagated in 17 cl1 cells. Assays for virus cell entry were done using recombinant MHV A59 virus, rA59EFLM, which has a firefly luciferase (FL) reporter gene insertion between the E and M open reading frames [48].

**Plasmid DNAs:** Plasmids used in this study include: pNL4.3-Luc R-E (obtained from the NIH AIDS Research and Reference Program), encoding the HIV-1 strain NL4 genome with firefly luciferase gene inserted into the pNL4-3 nef gene and expressing luciferase as marker of gene expression[49]; pcDNA 3.1 SARS S, encoding SARS Spike,
obtained from Michael Farzan, Harvard Medical School, Boston, MA; pHEF-VSV G encoding VSV glycoprotein G, from Lijun Rong, University of Illinois Chicago, Chicago; pcDNA 3.1 CEACAM, encoding CEACAM, the receptor for murine coronaviruses; Empty vector pCAGGS MCS[50]; pCAGGS-TMPRSS2-FLAG, pCAGGSTMPRSS11A-FLAG, pCAGGSTMPRSS11D-FLAG, encoding FLAG-tagged TMPRSS2, TMPRSS11A and TMPRSS11D, respectively[38]; pCAGGS A59 S, pCAGGS A59 E, pCAGGS A59 M, pCAGGS A59 N, encoding MHV A59 S, E, M and N proteins, respectively.

**Pseudo – coronavirus production:** 293T cells, seeded onto 6-well plates at ~50% confluency, were transiently transfected by the calcium phosphate precipitation method [51, 52], with pCAGGS MCS empty vector, or with 0.001µg/0.003µg/0.01µg/0.03µg/0.1µg/0.3µg/1µg of pCAGGS-TMPRSS2-FLAG and 1µg each of pNL4-3LUC-R-E, and pHEF-VSVG/pcDNA3.1-S-SARS. 48 hours post-transfection (h.p.t), supernatants containing secreted pseudoviruses were collected and clarified by centrifugation at 1000xg for 10min at 4 °C. For Western blot analysis, the pseudoviruses were purified by ultracentrifugation over a 30% sucrose cushion at 55000rpm for 1hour and 15min at 4 °C using a Beckman SW60 rotor. The pelleted pseudovirus particles were resuspended in SDS-DTT solubilizer (0.125 M Tris [pH 6.8], 10% dithiothreitol [DTT], 2% sodium dodecyl sulfate [SDS], 10% sucrose, 0.004% bromophenol blue) and heated at 95 °C for 5min.

**Pseudo – coronavirus transduction:** For pseudovirus transduction assays, 96-well plates were seeded with 293 ACE2 cells at a density of 4 x10⁴ cells /well, infected
with the pseudoviruses, and spinoculated at 3000rpm for 90 min at 25°C. Fresh media was added back to the wells and the plates were incubated at 37°C. After 48 hours transduction, the target cells were lysed with Promega reporter lysis buffer (Cat# E397A) and luminescence measured upon addition of Promega Luciferase substrate (Cat#E1501) with a Veritas microplate luminometer.

**Production of MHV Virus-Like-Particles (VLPs):** 293T cells seeded onto 6-well plates were transiently transfected with 1µg each pCAGGS A59 S, E, M, N and MCS empty vector, with or without additional 0.1/1µg of pCAGGS-TMPRSS2-FLAG. The calcium phosphate precipitation method was used for transfection [51, 52].48 h.p.t., supernatants containing secreted VLPs were collected and clarified by centrifugation at 1000xg for 10min at 4C. The VLPs were purified by ultracentrifugation through a 30% sucrose cushion at 55000 rpm for 1 hour and 15min at 4 °C with a Beckman SW60 rotor. The pelleted VLPs were resuspended in SDS-DTT solubilizer and heated at 95 °C for 5 min for subsequent evaluation by Western Blotting.

**Production of rA59EFLM from cells transiently expressing TMPRSS2:** 293T cells seeded onto 10cm-diameter dishes at >60% confluency were transfected with 1µg each of pcDNA mCEACAM and pCAGGS MCS empty vector, with or without 0.01/0.1/1µg pCAGGS TMPRSS2-FLAG. The Polyethylenimine (PEI) transfection method was used [53]. 24 h.p.t, the cells were infected with rA59EFLM at an M.O.I of 0.01. Viruses secreted in the supernatant were collected (5ml/dish) ~20 hours post-infection (h.p.i.), clarified for 10min at 2000xg, and used for infectivity assays. Viruses in 1ml of each clarified media were ultracentrifuged through 30% sucrose at 55000rpm for
1 hour and 15 min at 4 °C using a Beckman SW60 rotor. The pelleted viruses were resuspended in SDS-DTT solubilizer and heated at 95 °C for 5 min for subsequent evaluation by Western Blotting.

**MHV2 production in cells transiently expressing TMPRSS2:** 293T cells seeded onto 6-well (10 cm² per well) plates at ~50% confluency were transiently transfected with 1 µg pcDNA3.1CEACAM and pCAGGS MCS empty vector, or , 0.01 µg/0.1 µg/1 µg of pCAGGS-TMPRSS2-FLAG, by the calcium phosphate precipitation method. 24 h.p.t, the cells were infected with MHV2 virus (3x10⁸ pfu/ml) at a multiplicity of infection (MOI) of 1. Viruses secreted in the supernatant (1.5 ml/well) were collected 20 hrs post-infection, and clarified by centrifugation at 1000xg for 10 min at 4 °C, 100 µl volumes of each clarified supernatant were used for plaque assay titration and 1 ml volumes were ultracentrifuged over 30% sucrose cushions at 55000 rpm for 1 hour and 15 min at 4 °C using a Beckman SW60 rotor. The pelleted viruses were resuspended in SDS-DTT solubilizer and heated at 95 °C for 5 min for subsequent evaluation by Western Blotting.

**MHV A59 infectivity assay in the presence of drugs:** HeLa-CEACAM cells seeded onto 96 well plates at ~80% confluency were incubated with either complete DMEM media as control or 20 nM Bafilomycin A1 (Sigma Aldrich Cat #B1793) in complete DMEM media for 30 minutes at 37 °C. The cells were then treated with either 50 µM Aprotinin (Sigma Aldrich Cat # A6279), or DMEM and infected with rA59EFLM viruses that had been produced in cells expressing graded amounts of TMPRSS2, and the infection was allowed to continue in the presence of the inhibitors. After 6 hours of
incubation at 37°C, the infected target cells were lysed to measure luciferase accumulations.

**Plaque assay titration of MHV2 virus produced in cells transiently expressing TMPRSS2:** 17C11 cells seeded on 6-well plates were infected with 100µl volumes of MHV2 that had been serially diluted in serum free media. After 1 hour of infection at 37 °C, the infection medium was aspirated and the cells were overlaid with 2ml/well of 1% Noble agar mixed with 2xDMEM containing 2% FCS and incubated at 37°C. After 48 hours, the cells were fixed in 25% Formalin, stained with Crystal violet, and plaques were counted.

**Western blot analysis:** Purified HIV pseudoviruses (HIV SARS S, HIV A59 S and HIV VSV G), MHVs (MHV2 and rA59EFLM) and MHV VLPs ,resuspended in SDS-DTT solubilizer were heated at 95°C for 5min and subjected to SDS-polyacrylamide gel electrophoreses (SDS-PAGE). The gels were transferred on to polyvinylidene difluoride (PVDF) membranes which were then blocked for 1 hour with 5% non-fat milk powder in TBST (25 mM Tris-HCl {pH 7.5}, 140 mM NaCl, 2.7 mM KCl, 0.05% Tween 20). HIV p24 proteins were detected with mouse monoclonal anti-p24 antibody (NIH AIDS Research and Reference Program) diluted 1:5000 in TBST. SARS Spike was detected with anti-C9 tag (1D4) antibody (1:5000 in TBST). MHV2 and MHV A59 spike proteins were detected using anti S2 (10G) mouse monoclonal antibody [15, 54] (1:2000 in TBST). MHV A59 M proteins were detected using mouse monoclonal anti-M J.3.1[55] antibody(1:500 in TBST).
The effects of TTSPs on pseudovirus transduction:

Of the 17 known human TTSPs[39], TMPRSS11A and TMPRSS11D (Human Airway Trypsin-like protease) and TMPRSS2 were tested for their effects on HIV-SARS assembly because these three are expressed in the respiratory tract and have been shown to proteolytically activate Influenza Hemagglutinin, Human Metapneumovirus F proteins and SARS-CoV Spike-mediated entry[36, 37, 41, 44]. HIV-pseudotyped vectors expressing the luciferase reporter gene[56] and bearing SARS-CoV S proteins were produced in 293T cells that had been transiently transfected to express varying amounts of TMPRSS proteins. The pseudoviruses thus produced were used to transduce 293T cells that stably express ACE2. 2 days post-transduction, the target cells were lysed and luciferase accumulations were measured as readout for pseudovirus entry. Figure 3A presents a schematic diagram of this approach.

The presence of TMPRSS 11A, TMPRSS11D and TMPRSS2 in the pseudovirus producing cells had an inactivating effect on the transducing abilities of HIV SARS pseudoviruses, with the effect of TMPRSS2 being most potent(Fig. 3B), hence, further study of TTSPs was pursued with TMPRSS2.
When co-expressed in HIV-SARS and HIV-VSVG producing cells, increasing amounts of TMPRSS2 specifically inhibited HIV-SARS S-mediated transduction, while the transducing ability of HIV-VSVG was relatively unaffected (Fig. 4). The inhibition of transduction observed with HIV-SARS S also extended to another CoV pseudotype, HIV–MHV A59 (Fig. 5).

**Figure 3A. Schematic diagram depicting pseudovirus production and transduction:** 293T producer cells were co-transfected with 1 µg each of plasmids encoding HIV-Luciferase, SARS S and empty vector or 0.01µg/0.1µg/1µg TMPRSS2, TMPRSS11A and TMPRSS11D.
and TMPRSS11D. Pseudoviruses collected 48 h.p.t were used to transduce 293-ACE2 cells.

Figure 3B. Effect of TTSPs 2, 11A and 11D on HIV-SARS S transduction: Luciferase values measured 2 days post-transduction were plotted on a Log_{10} scale. The error bars represent standard deviations.
Figure 4. TMPRSS2 dose-response: Transduction abilities of HIV-SARS S and HIV- VSV G pseudoviruses produced in cells expressing graded amounts of TMPRSS2: HIV-SARS S and HIV-VSV G pseudoviruses were produced by co-transfecting 293T cells with HIV vector, HIV-SARS S or HIV-VSV G vector and pCAGGS Empty Vector or the indicated amounts of pCAGGS TMPRSS2-FLAG. 48 h.p.t, media containing the pseudoviruses were collected and used to transduce target 293T-ACE2 cells. Target cells were lysed 2 days post-transduction and luciferase accumulations were measured. The error bars represent one standard deviation above and below the mean.
Figure 5. Transduction ability of HIV-MHV A59 S pseudoviruses produced in cells expressing TMPRSS2: HIV-A59 S pseudoviruses were produced by co-transfecting 293T cells with HIV vector, MHV A59 S vector and pCAGGS Empty Vector or the indicated amounts of pCAGGS TMPRSS2-FLAG. The pseudoviruses were used to transduce target HeLa-CEACAM cells which were lysed 2 days post-transduction and measured for luciferase accumulations. The error bars represent one standard deviation above and below the mean.

The effect of TMPRSS2 on HIV SARS S and HIV A59 S protein cleavage:

The dramatic reduction in transduction potential mediated by TMPRSS2 (Figs. 4 and 5) suggested that TMPRSS2 might degrade CoV S proteins during pseudovirus assembly and/or secretion.

To test this hypothesis, the HIV-SARS S and HIV-MHV A59 S pseudoparticles that were evaluated for transduction potential (Figs. 4 and 5) were purified by ultracentrifugation and evaluated for S protein cleavage by Western immunoblotting.
The data (Fig. 6) revealed that both SARS and MHV A59 S proteins were degraded with increasing doses of TMPRSS2, with the degree of degradation corresponding to the decreases in transducing abilities.

**Figure 6. S cleavage on HIV-SARS S and HIV-A59 S pseudoviruses:** HIV SARS S and HIV A59 S pseudoviruses produced in 293T cells transfected with the indicated amounts of pCAGGS TMPRSS2-FLAG were purified by ultracentrifugation. Resuspended pseudoviruses were evaluated by western immunoblotting for HIV p24 (anti-p24 antibody) and SARS S (anti-C9 antibody) or MHV A59 Spike (10G anti-S2 antibody)

**The effect of TMPRSS2 on MHV VLP production:**

HIV pseudoviruses bud at the plasma membrane, where TMPRSS2 is expressed [57, 58], whereas authentic coronaviruses bud intracellularly at the ER-Golgi-Intermediate-Compartment (ERGIC) [9, 59]. The degradation of HIV–CoV spikes observed in Fig. 6 could have occurred during pseudovirus budding from the cell surface,
where the spikes may be readily accessible to TMPRSS2. To determine if the site of spike assembly plays a role in determining its susceptibility to the protease, I utilized MHV A59 virus-like-particles (VLPs), and compared the effect of TMPRSS2 on spikes that were either assembled on the plasma membrane or intracellularly at the ERGIC. MHV S proteins, when expressed alone, are transported to the plasma membrane, while the co-expression of MHV structural proteins S, E, M and N can result in the self-assembly of VLPs at the ERGIC [60, 61]. MHV VLP assembly mimics authentic virus assembly and the secreted particles resemble authentic viruses, but the VLPs are not infectious because they do not express viral genetic materials. This property makes the VLPs a good system to more accurately assess coronavirus assembly in cells expressing TMPRSS2.

Briefly, 293T cells were co-transfected with plasmids encoding TMPRSS2 and either MHV A59 S alone, or S in combination with E, M and N. Cell lysates (expressing S alone) and secreted VLPs were evaluated for spike cleavage by immunoblotting.

The data (Fig. 7) revealed that spike proteins on MHV VLPs were degraded by TMPRSS2, similar to the S degradation expressed alone in cells. The susceptibility of MHV VLP spikes to TMPRSS2-mediated cleavage suggests that spikes are degraded during their assembly in the exocytic pathway. Indeed, both TMPRSS2 and the S proteins are thought to co-exist in the ER, Golgi and secretory vesicles comprising the exocytic pathway. Possibly S cleavages take place internally in one or more of these subcellular compartments.
Figure 7. **S cleavage on MHV A59 VLPs:** MHV A59 VLPs were produced by co-transfecting 293T cells with MHV A59 S, E, M, N vectors and pCAGSS Empty Vector or the indicated amounts of pCAGGS TMPRSS2-FLAG. VLPs produced were purified by ultracentrifugation and evaluated by Western Immunoblotting for MHV A59 S and M proteins with 10G (anti-S2) and anti-M J3.1 antibodies.
CHAPTER FOUR
TMPRSS2 AND AUTHENTIC CORONAVIRUS ASSEMBLY

Infectivity of MHV A59 viruses produced in TMPRSS2-expressing cells:

Data in the previous chapter revealed that HIV-CoV pseudoviruses secreted from cells expressing TMPRSS2 have greatly diminished transduction potential. The effect of TMPRSS2 on the production of authentic coronaviruses (murine coronaviruses rA59EFLM and MHV 2) was investigated next. Here we used rA59EFLM, which encodes the firefly luciferase gene within its genome, making assay of the luciferase accumulated in infected target cells a good measure of viral entry and infectivity.

Fig.8A illustrates the overall experimental approach. Briefly, 293T cells that were co-transfected to express CEACAM (the MHV receptor) and TMPRSS2 were subsequently infected with rA59EFLM viruses. Progeny viruses secreted in the media were collected ~20 h.p.i and used to infect HeLa-CEACAM target cells. Luciferase accumulations were measured in the target cells at 6 hours post-infection to assess virus entry.

Although the effect of TMPRSS2 on authentic MHV infectivities (Fig.8B) was not as dramatic as its effect on HIV A59 pseudovirus transduction, there was about a 2-log reduction in virus infectivity. The data indicated that viruses exiting cells over-expressing TMPRSS2 have reduced infectivities.
Fig 8A. Schematic diagram of production and infectivity assay of rA59EFLM viruses in the presence of TMPRSS2: 293T cells were co-transfected with plasmids encoding murine CEACAM receptor and empty vector or 0.01µg/0.1 µg/1 µg TMPRSS2. 24 h.p.t, the cells were infected with rA59EFLM at an M.O.I of 0.01. Viruses secreted in the supernatant were collected 20 h.p.i. and used to infect target HeLa-CEACAM cells.
Figure 8B. Infectivity of rA59EFLM viruses produced in cells expressing TMPRSS2: Luciferase values measured 6 h.p.i in HeLa-CEACAM target cells were plotted on a Log$_{10}$ scale. The error bars represent one standard deviation above and below the mean.

MHV S cleavage by TMPRSS2:

The reduced infectivity of rA59EFLM produced from TMPRSS2-expressing cells (Fig. 8B) suggested that S proteins were degraded during virus maturation. To determine if that was the case, rA59EFLM viruses were purified by ultracentrifugation and evaluated for S cleavage by Western immunoblotting (Fig. 9). Spike was detected by 10G antibody which recognizes the HR2 region of S2, as depicted in Figure 9A.

The data in Figure 9B revealed that increasing amounts of TMPRSS2 in producer cells degraded S proteins, in correlation with decreased infectivities observed (Fig. 8B). The viruses contained spikes that had been cleaved into ~80kDa, 72kDa, 70kDa and
50kDa C-terminal fragments. An arginine at the P1 position is required for TMPRSS2 cleavage [39] and there are six arginine residues in the ~250 amino acid region between S1/S2 and FP in the MHV S proteins. The 80kDa, 72kDa and 70kDa fragments observed in Fig 9B correspond to TMPRSS2-mediated cleavages at sites (colored arrows in Fig 9A) lying between the well-known S1/S2 cleavage site (white arrow in Fig. 9A) and the FP (black bar in Fig. 9A) and the 50kDa fragment corresponds to cleavage at a site C-terminal to the FP.

![Image of diagram](image-url)
Figure 9. MHV A59 S cleavage by TMPRSS2: (A) Schematic structure of spike: S protein has a signal peptide (SP), receptor binding domain (RBD), a fusion peptide (FP), two heptad repeats (HR1 and HR2), a transmembrane domain TM and a cytoplasmic tail (CT). The S1/S2 cleavage site is depicted by the white arrow and the black arrow depicts the epitopes recognized by the 10G(anti-S2) antibody. The arginine residues between the S1/S2 cleavage site and the FP are indicated. The possible TMPRSS2 cleavage sites resulting in the p80, p72, p70 and p50 fragments are indicated with colored arrows. (B) rA59EFLM viruses produced from 293T cells that were co-transfected with 1µg of pcDNA mCEACAM and the indicated amounts of pCAGGS TMPRSS2-FLAG or pCAGGS Empty Vector were purified by ultracentrifugation. Resuspended viruses were evaluated by western immunoblotting for S (10G antibody) and M (anti-M antibody).

MHV A59 activation by TMPRSS2 in virus-producing cells:

Recent reports [62-64] have indicated that the CoV S1/S2 cleavage may be insufficient for fusion activation, and that the S1/S2 cut is followed by a second cleavage at a site next to the fusion peptide, termed the S2′ cleavage site. This S2′ cleavage is thought to be the most biologically relevant scission, because it lies immediately N-terminal to the fusion peptide and thus would put the fusion peptide at the extremity of the transmembrane cleavage product. Thus the S2′ cleavage would make the CoV S proteins much like the traditional class I membrane fusion proteins that have their FPs at termini. The 70kDa (Fig.9B) cleavage fragments observed on rA59EFLM spikes from TMPRSS2-expressing cells could correspond to such fragments cleaved at the vicinity of the fusion peptide, suggesting that a portion of the viruses expelled from TMPRSS2-expressing cells may have “pre-activated” spikes ready to be inserted into target cell membranes. Entry into cells by such viruses may be insensitive to inhibitors of endosomal or cell surface proteases. To test this hypothesis, rA59EFLM viruses produced from TMPRSS2-expressing 293T cells were used to infect target cells in the presence of aprotinin (a membrane impermeable serine protease inhibitor [37, 65]), and/or
bafilomycin A1 (an ATPase inhibitor which prevents the endosomal acidification required for activity of some endosomal proteases [4, 66, 67]). Entry achieved by these viruses was compared to the entry of viruses produced from cells (293T and 17Cl1) that were not transfected with TMPRSS2 plasmids.

The results (Figs. 10A and B) revealed that MHV A59 viruses produced in cells expressing TMPRSS2 were relatively resistant to aprotinin and bafilomycin. In the presence of aprotinin, control viruses, produced from cells lacking exogenous TMPRSS2, were inhibited by ~160-fold (Fig. 10A) to 1000-fold (Fig. 10B) while the viruses produced from cells overexpressing TMPRSS2 cells showed only 2-fold inhibition. This indicated that MHV A59 viruses secreted from cells in the absence of TMPRSS2 are not fully activated for entry and require target cell proteases (endosomal proteases or cell surface serine proteases) for further activation, whereas TMPRSS2, in virus producer cells could provide the activating proteolysis required for entry.
Figure 10A. Effect of Bafilomycin and Aprotinin on the entry of rA59EFLM viruses that were produced in the presence of graded amounts of TMPRSS2:
rA59EFLM viruses that had been produced in 293T cells in the presence of the indicated amounts of pCAGGS TMPRSS2-FLAG were used to infect target HeLa-CEACAM cells in the presence of complete DMEM media (No drugs) or 20nM Bafilomycin A1 and/or 50µM Aprotinin. Cells were lysed 6 h.p.i and luciferase accumulations were measured. Error bars represent one standard deviation above and below the mean.
Figure 10B. Effect of Bafilomycin and Aprotinin on rA59EFLM entry:

rA59EFLM viruses that were produced in 17Cl1 cells in the absence of TMPRSS2 were used to infect HeLa-CEACAM cells in complete DMEM media (No Treatment) or 20nM Bafilomycin A1 and/or 50µM Aprotinin. Cells were lysed 6 h.p.i and assayed for luciferase accumulations. Error bars represent one standard deviation above and below the mean.

Thermal inactivation of MHV A59 viruses produced in TMPRSS2-expressing cells:

Stability of S1-S2 interactions have been correlated to thermal stability of some strains of MHV, specifically, the neurovirulent MHV JHM.San Diego (JMH.SD)[68]. JHM.SD spikes are cleaved at the S1-S2 junction. The non-covalent interaction between the two subunits is dissolved by elevated pH at the cell surface, and S1 dissociates spontaneously from S2 [69, 70], rendering the virus in a fusion-competent state. The dissociation of S1 from S2 correlates with enhanced neurovirulence, and also tissue
culture instability [8]. Evidence of rA59EFLM spike cleavage by TMPRSS2 at positions near the fusion peptide (Fig. 9B, note ~ 70 kDa bands) suggested that these extra cleavages might render the viruses thermally labile, similar to the JHM.SD viruses. To test this hypothesis, rA59EFLM viruses were incubated at 37°C for 5, 10, 20, 40, 80 min, and then used to infect target HeLa-CEACAM cells. At 6 hpi, target cells were lysed to measure luciferase accumulations.

The data (Fig. 11) indicated that the MHV A59 viruses expelled from cells expressing TMPRSS2 were indeed hyperlabile. Perhaps the preactivated viruses with cleaved spikes undergo more rapid intrinsic denaturation over time, resulting in rapidly declining infectivities.
Figure 11. Thermal inactivation of rA59EFLM viruses produced in the presence of TMPRSS2: rA59EFLM viruses that were produced in the presence of the indicated amounts of pCAGGS TMPRSS2-FLAG were incubated at 37°C for 0, 5, 10, 20, 40 and 80 minutes. The viruses were then used to infect HeLa-CEACAM cells. Cells were lysed 6 h.p.i to assay for luciferase accumulations.

Infectivity and Spike-cleavage of MHV2 viruses produced in TMPRSS2-expressing cells:

MHV2 spikes, like SARS-CoV spikes, are produced uncleaved during assembly and secretion from infected cells. Production of MHV2 viruses in TMPRSS2-expressing cells could give insight to the infectivity of SARS-CoVs produced under similar conditions.

To test the effect of TMPRSS2 on MHV2 production, MHV2 viruses were used to infect 293T cells that were transfected to express murine CEACAM receptor and TMPRSS2. Progeny viruses secreted in the media were collected 20 hpi and titered on 17Cl1 cells to assay infectivity. Purified viruses were evaluated for S cleavage by Western Immunoblotting using anti-S2 (10G) antibody, which recognizes epitopes in the HR2 region of the C-terminal S2 subunit (illustrated in Fig. 12C).

The results (Fig. 12) revealed that MHV2 viruses produced in the presence of TMPRSS2 were about 20-fold less infectious than control viruses that were produced in the absence of the protease. The presence of TMPRSS2 in producer cells seemed to result in an inactivating cleavage of MHV2 spike at a site C-terminal to the fusion peptide (red arrow in Figure 12C), to a ~55kDa product corresponding to the protease-resistant core of the six-helix bundle conformation (6HB) formed during membrane fusion events.
Figure 12. Effect of TMPRSS2 on the infectivity and Spike cleavage of MHV2 viruses: MHV2 viruses were produced in 293T cells that were co-transfected with 1µg of pcDNA mCEACAM and the indicated amounts of pCAGGS TMPRSS2-FLAG or pCAGGS Empty Vector. A) Viruses secreted in the media were clarified by centrifugation and titered on 17Cl1 cells by plaque assay. B) Viruses were pelleted by ultracentrifugation, resuspended, and evaluated by western immunoblotting for MHV2 Spike protein (10G anti-S2 antibody). C) Schematic depiction of MHV2 spike [15] with the 10G antibody recognition site (black arrow) and the putative TMPRSS2 cleavage site (R939, red arrow) C-terminal to the fusion peptide (FP).
CHAPTER FIVE

CONCLUSION

Coronavirus spike is an important determinant of viral tropism. The spatiotemporal sequence of spike cleavage is extremely important to viral pathogenicity and the proteolytic processing that spike undergoes can differ between different coronaviruses. SARS coronavirus spike is synthesized uncleaved and during entry; after receptor attachment the spike undergoes sequential cleavage at the S1-S2 junction (Fig.13, “1”), followed by cleavage at the S2 region (Fig 12.” 2”) [62]. The S2 cleavage exposes its internal fusion peptide which gets inserted to the target cell membrane [63].

![Fig.13. Schematic depiction of sequential proteolytic cleavage of SARS CoV spike[62]: Cleavage at the S1/S2 boundary facilitates cleavage at the S2 position exposing the fusion peptide which can then be inserted into the target membrane for membrane fusion to occur.(FP: Fusion peptide; HR1: Heptad repeat 1; TM: Transmembrane domain)](image)

MHV2, like SARS CoV, emerges from cells with uncleaved spikes. Engagement of the virus with CEACAM on a target cell, triggers stepwise conformational changes in spike[15, 24] which begin with the exposure of the viral fusion peptide. The insertion of the fusion peptide into the cell membrane results in a pre-hairpin trimer with one end
embedded in the cell membrane and the other in the viral envelope. Protease-mediated cleavage results in a fusogenic fragment containing the fusion peptide, HR1 and HR2 and the transmembrane domain, triggering the next step of conformation changes. Formation of the 6-helix bundle (6HB), characterized by a proteinase K-resistant core appearing as a ~53kDa band in western blot images[15], draws the two membranes close together resulting eventually in membrane fusion as depicted in Figure 2.

MHV2 viruses produced in TMPRSS2 expressing cells are less infectious (Fig12, left panel) but the cleavage pattern of spikes on these viruses is different from that observed in MHV A59 spikes. In producer cells that do not express TMPRSS2, MHV2 spike is synthesized and assembled in an uncleaved form on the emerging virion but when producer cells express TMPRSS2, the spikes on the emerging viruses are cleaved and appear as a ~55kDa band (Fig 12, right panel) which corresponds to the proteinase-K resistant core of the 6-helix bundle (6HB) usually formed during fusion events after the interaction of spike with its receptor. It is possible, that as in the case of SARS Spike, the timing of MHV2 spike cleavage is crucial in virus infectivity. Spike cleavage in MHV2 during virus production probably occurs in the absence of receptor engagement and hence could be an inactivating cleavage event. But, during MHV2 entry, when the virus is bound to its cell-surface receptor, the ensuing conformation changes of spike could expose previously hidden, potentially activating TMPRSS2-cleavage site(s). Cleavage at the newly exposed site(s) of spike by TMPRSS2, expressed on the virus-target cell, could therefore possibly be an entry-activating event.
Unlike MHV2 and SARS-CoV, MHV A59 spikes are synthesized precleaved at S1/S2 during biogenesis. This study reveals that the intracellular MHV A59 S cleavage is not the only activating step required for successful infection (Fig 10A and B). Spike requires further cleavage, perhaps at or near the S2 region, by either cell surface serine or endosomal acidophilic proteases, since inhibition of these proteases resulted in a decrease in viral entry of up to a 1000-fold. The extents of inhibition by either the serine protease inhibitor aprotinin or the endosomal protease inhibitor bafilomycin further suggests that on entering target cells, the viruses can undergo two different, independent activation pathways. About two-thirds of the entering viruses can get activated at cell surfaces by serine-proteases that are inhibited by aprotinin. The other one-third of the entering viruses do not get activated in this way and are then activated in endosomes by acidophilic proteases, likely cathepsins, that are inhibited by bafilomycin. Either of the two activation processes are sufficient for virus entry – the aprotinin-sensitive activation does not require additional bafilomycin-sensitive activation and vice versa. The action of these proteases could result in cleavage at the S2 region, exposing the fusion peptide for subsequent insertion into target cell membranes as depicted in Fig.14.
Figure 14. Model for MHV A59 entry effects of proteases on target cells: Figure modified from “Role of Spike Protein Endodomains in Regulating Coronavirus Entry” Shulla, A., et al., JBC, 2009[31]. Conformation change occurring upon receptor binding by the S1 subunit of MHV spike exposes the S2 cleavage site to the action of target cells proteases. Cleavage at the S2 site (middle panel) exposes the fusion peptide (FP) which is inserted into the cell membrane forming the prehairpin trimer intermediate.

Viruses emerging from TMPRSS2 expressing cells were less stable (Fig 11), had lower infectivities (Fig 8B) and appeared to be relatively resistant to aprotinin (Fig. 10A). It is possible that a portion of the viruses secreted from TMPRSS2 expressing cells bear spikes that have been cleaved at the S2 cleavage site and have their fusion peptides exposed and pre-primed for target cell membrane insertion. The 70kDa S2 fragments that appear in viruses from TMPRSS2 positive cells (Fig 9) could correspond to the pre-primed S2 fragments which do not need further activation by target cell proteases and hence are insensitive to protease inhibition. On the other hand, while some viruses are secreted with pre-primed spikes, it appears that another fraction of the progeny virions have inactivating spike cleavage with S2 fragments ~50kDa in size which also corresponds to the overall lowered infectivities of viruses emerging from TMPRSS2 expressing cells.
Fig. 15 depicts a model for the effects of TMPRSS2 protease activity on MHV production. In an infected cell, progeny MHV virion morphogenesis occurs by budding at the ERGIC. It is possible that during maturation and egress through the compartments of the host cell secretory pathway, the viruses come in contact with newly synthesized TMPRSS2 proteins being transported to the plasma membrane. The proximity of protease and substrate within the same intracellular compartments likely facilitates TMPRSS2-mediated cleavage of accessible spikes into the ~ 80kDa, 72kDa, 70kDa and 50kDa fragments observed in the western blot image in Fig 9B. It is not clear, however, whether the TMPRSS2-mediated cleavage affects the non-covalent interactions between S1 and S2 and whether the cleaved spikes on the expelled viruses are maintained as stable S1-S2 complexes.
Figure 15. Model for TMPRSS2 effects on MHV production: In an infected cell, nascent MHV virions and newly synthesized TMPRSS2 proteins are transported together to the cell surface by secretory vesicles of the exocytic pathway. Transport in the same intracellular compartments renders spike proteins on the virions accessible to the proteolytic activities of TMPRSS2 and viruses emerge from the cells bearing cleaved spikes.
It is conceivable that cleavage by TMPRSS2 in MHV-producer cells results in the loss of segments of spike that are important for stable S1-S2 interaction. The decreased thermo-stability observed in these viruses (Fig.11) could be a consequence of a decreased stability in S1-S2 interactions causing spontaneous S1 detachment not unlike that observed in MHV JHM.SD [8, 68, 69].

Proteases play a crucial role in coronavirus spike activation and the timing and location of the cleavage can make the difference between an activating and an inactivating cleavage. Unlike influenza and HMPV viruses, which are activated in producer cells by TMPRSS2 mediated cleavage of their precursor glycoproteins during transit through the cellular exocytic pathway, MHV coronaviruses emerging from TMPRSS2-expressing cells are overall less infectious and are subject to inactivating spike cleavage. The “pre-priming” of some MHV A59 viruses by TMPRSS2 in the producer cells is balanced by the inactivating spike cleavage of some emerging viruses.

The findings from this study have implications for the study of host cell factors that play a role in coronavirus infection and evolution. Collectively, the data indicate that the coronavirus entry activation that is facilitated by TMPRSS2 [38] might be tempered by the subsequent inactivation of progeny viruses by the proteases, possibly as a mechanism of host cell resistance. Development of host cell resistance can necessitate co-evolution of viruses to select for more virulent variants as a mechanism to establish persistent infection [71-73]. It is conceivable that serial passaging of coronaviruses in cells expressing TMPRSS2 could result in the emergence of mutations in the inactivating
cleavage sites recognized by the protease thus producing more virulent TMPRSS2-resistant viruses
APPENDIX

EFFECT OF ANTI VIRAL LECTIN GRIFFITHSIN ON CORONAVIRUS INFECTION
Griffithsin (GFRT) is a mannose binding plant lectin with potent anti-viral properties that was isolated from red algae Griffithsia in New Zealand in 2004, during an NCI, National Institutes of Health sponsored search for natural product extracts exhibiting anti-HIV activity [75]. Lectins are commonly occurring proteins in nature that bind carbohydrates and play varied roles in cellular processes. The propensity of lectins to bind carbohydrates makes them attractive candidates for antiviral agents since enveloped viruses such as retroviruses and coronaviruses are embedded with heavily glycosylated surface glycoproteins, which when targeted can lead to disruption of the virus-host cell interactions and inhibit virus entry[74].

GFRT binds to oligosaccharides on HIV gp120 and inhibits cytopathic effects of HIV-1 at picomolar concentrations, without having cytotoxic effects on cells at high concentrations, indicating its promise as a potential anti-viral microbicide [75].

A recent study indicates that GFRT mediated inhibition extends to coronaviruses and intranasal administration of the lectin prevents lethal pulmonary infection in mice [76]. The goal of this study was a better understanding of the mechanism of action of GFRT, which could aid in the development of preventative and therapeutic measures such as topical anti-viral microbicides.

**Methods**

**Cells and viruses**

The cell lines used in this study, 293T cells and HeLa-CD4 cells stably transfected to express CD4[77], were cultured in DMEM supplemented with 10%FBS, 2mM L-Glutamine, 100 units/ml penicillin, 100µg/ml streptomycin and 0.01M sodium HEPES (pH7.4). Murine 17c1l fibroblasts used to passage MHV A59 viruses were grown in media containing DMEM , 5% tryptose phosphate broth, 5% FCS and 0.01M sodium HEPES (pH7.4). SFM used in pseudovirus transduction and cell-cell fusion assays contained 2mM L-Glutamine, 100 units/ml penicillin, 100µg/ml streptomycin and 0.01M sodium HEPES (pH7.4).

**Plasmid DNAs**

Plasmids used in the study included, pNL4-3LUC-R-E( encoding the HIV-1 strain NL4 genome with firefly luciferase gene inserted into the pNL4-3 nef gene and expressing luciferase as marker of gene expression[49]), pcDNA 3.1 CEACAM (encoding CEACAM the receptor for MHVs), pHEF-VSV G (encoding VSV glycoprotein G), pCAGGS A59 S (encoding MHV A59 S) , pCAG T7pol (encoding T7 RNA polymerase), pT7EMC-luc (encoding firefly luciferase under the control of T7 RNA polymerase promoter).

**Pseudovirus transductions**

HIV-pseudotyped virions were produced by co-transfecting 293T cells,
via calcium phosphate, with pNL4-3LUC-R-E, and plasmid vectors encoding MHV A59 S/VSV G/gp120/41 from HIV-1 JR-FL strain. Pseudovirions secreted in the media were collected 48 hpi, clarified at 2000xg for 10min, 4°C and stored at -20°C. For transductions, target HeLa-CD4 cells were transfected, via lipofection[78] (using Lipofectamine™ 2000 transfection reagent, Invitrogen), with pcDNA CEACAM. 24 hpt, the target cells were incubated with either SFM or 100nM, 200nM, 400nM Griffithsin (obtained from the NIH AIDS Research and Reference Program) in SFM, for 20min at 22°C. After 20min, the media was aspirated, pseudovirions were added and the plates were spinoculated by centrifugation at 25°C for 2 hours at 3000rpm. After 2 days of transduction at 37°C, the target cells were lysed and luciferase activity measured.

**Cell-cell fusion assay**

Target 293T cells were co-transfected with pcDNA3.1 CEACAM and pT7EMC-luc via calcium phosphate. Effector cells were produced by co-transfecting 293T cells with pCAGGS A59 S and pCAG-T7pol via calcium phosphate. 28 hours post transfection, effector cells were resuspended in serum free media (SFM) and overlaid on the target cells at a 1:1 ratio and spinoculated at 500xg for 5min, at 4°C.100nM, 200nM, 400nM Griffithsin in SFM, or SFM alone, was added and the cells were incubated at 37°C. After 4 hours, the cells were lysed and assayed for luciferase accumulations.

**Immunoprecipitation and immunoblotting**

nCEACAM-Fc proteins[23] and protein G magnetic beads were resuspended in HN buffer (25 mM Na Hapes, 150 mM NaCl )and incubated at 22°C for 1 hour. The beads were rinsed thrice with HNB buffer (25 mM Na Hapes, 150 mM NaCl, 0.01% BSA) containing 0.5% sodium deoxycholate, 0.5% NP40 to wash away unbound nCEACAM-Fc proteins. The beads were then incubated with SFM (No GFRT), or 100nM, 200nM, 400nM GFRT in SFM at 22°C for 20min.After rinsing thrice with HNB buffer, the beads were incubated with MHV A59 viruses at 37°C for 1 hour. The beads were rinsed thrice in HNB and the proteins were eluted from the beads by adding SDS-DTT solubilizer (0.125 M Tris {pH 6.8}, 10% dithiothreitol, 2% sodium dodecyl sulfate [SDS], 10% sucrose, 0.004% bromophenol blue), and heating at 95°C for 5min. The samples were subjected to 12% SDS polyacrylamide gel electrophoresis and the gels were transferred to polyvinylidene difluoride membranes which were then blocked for 1 hour with 5% non-fat milk powder in TBST (25 mM Tris-HCl {pH 7.5}, 140 mM NaCl, 2.7 mM KCl, 0.05% Tween 20). Spike proteins were detected using monoclonal, anti-S2 (10G) antibody (1:2000 in 2% milk in TBST), and GFRT was detected using anti-GFRT antibody (1:1000 in 2% milk in TBST).
Results
Effect of GFRT on pseudovirus transductions

The effect of GFRT on coronavirus infections was examined using HIV-pseudotyped MHV A59 S pseudoparticles in HeLa-CD4 cells transiently expressing CEACAM receptor. Briefly, HIV-A59 S, HIV-VSV G and HIV-JR-FL pseudoviruses were used to transduce HeLa-CD4 target cells in the presence of increasing amounts of GFRT. 48 hours post-transduction, the target cells were lysed and assayed for luciferase activities. HIV-JR-FL was used as a positive control, since the inhibitory effect of GFRT on HIV has been documented [75]. The results (Fig 16) revealed that the transduction ability of HIV-A59 S was decreased almost 10-fold by Griffithsin, and this effect was specific to coronaviruses since transduction potential of HIV-VSV G was not affected.

Figure 16. Effect of GFRT on HIV-MHV A59 S pseudovirus transduction: HeLa-CD4 cells transfected to express CEACAM receptor were incubated with 100nM, 200nM, 400nM GFRT in serum free media for 20min at 22°C. Media was removed and the cells were incubated with HIV-SARS S, HIV-VSV G and HIV-JR-FL for 2 days at 37°C. 48 hours post-transduction, cells were lysed and luciferase activity was measured. Error bars represent one standard deviation above and below the mean.

Effect of GFRT on virus: receptor interaction

To determine whether GFRT blocks virus-receptor interaction, a co-immunoprecipitation experiment was performed using an Fc-tagged,
soluble form of CEACAM, the receptor for MHV A59, nCEACAM-Fc [18, 23]. nCEACAM-Fc was captured on to protein G magnetic beads and incubated with or without GFRT. MHV A59 viruses were then added and after a one hour incubation period, proteins were subsequently eluted from the beads and subjected to immunoblotting to detect Spike proteins. The results revealed that GFRT bound CEACAM (Fig. 17, lane 5), but did not inhibit the subsequent binding of the virion with the receptor, even at higher concentrations (Fig 17, lanes 2, 3 and 4). The data indicates that the site of action of GFRT is not at the stage of virus-receptor binding.

**Figure 17: Effect of GFRT on virus-receptor binding:** nCEACAM-Fc, the soluble form of the receptor for MHV A59, bound to Protein A magnetic beads, was incubated with the indicated concentrations of GFRT (Griffithsin) at 37°C for 30 min, washed, followed by incubation with purified, wild type MHV A59 virus for 30 min at 37°C. The samples were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. Lanes 6 and 7 are controls to ensure that GFRT and MHV A59 are not binding the magnetic beads. Lanes 8 and 9 are GFRT and MHV A59 loading controls, respectively.
Figure 18. Effect of GFRT on MHV A59 Spike-mediated cell-cell fusion: Target cells (293T cells transfected with plasmids encoding CEACAM and Luciferase) were overlaid, 28hrs post-transfection, with Effector cells (293T cells transfected with plasmids encoding MHV A59 Spike protein and T7 Polymerase) at 1:1 ratio and spinoculated at 4 °C for 5min to facilitate Effector-Target cell contact without the initiation of fusion. The indicated concentrations of Griffithsin (GFRT) were then added, and the Effector-Target cells were co-incubated at 37°C for 4hrs, lysed and assayed for Luciferase activity. Error bars represent one standard deviation above and below the mean.

Effect of GFRT on MHV A59 Spike mediated cell-cell fusion

Data from Fig 17 indicated that GFRT may be exerting its MHV A59 entry inhibiting effects at post-receptor-binding stages. To determine if that was the case, MHV A59 S mediated cell-cell fusion assay was performed in the presence of GFRT which was administered after ensuring spike-CEACAM binding. Briefly, target cells were co-transfected with plasmids encoding CEACAM and a luciferase reporter under the control of T7 RNA polymerase promoter. Effector cells were co-transfected to express MHV A59 S, and T7 RNA polymerase. At 28 hpt, the target cells were overlaid with effector cells at a 1:1 ratio and spinoculated to facilitate spike-CEACAM binding. The spinoculation was performed at 4 °C, a temperature that arrests receptor-mediated spike conformational changes and membrane fusion [24]. GFRT was then added to the cells and the reaction shifted to 37 °C to allow for cell-cell fusion. Cells were lysed after a 4 hour co-culture and assayed for luciferase accumulations. As shown in Figure 18, GFRT inhibited MHV A59 Spike mediated cell-cell fusion in a concentration dependent manner. In this experiment, the effector cells
bearing the MHV Spike protein were overlaid on the CEACAM receptor-bearing target cells and spinoculated at 4°C (to ensure spike-receptor binding without fusion initiation), followed by the addition of increasing concentrations of GFRT and shift to 37°C. The cell-cell fusion decreased to ~60% of the “No treatment” control in the presence of 100nM GFRT and up to 40% in the presence of 200nM and 400nM GFRT. The result indicated that the cell-cell fusion blocking effect of GFRT takes place after virus: cell receptor interaction. Collectively, figures 17 and 18 suggested that the inhibitory effect of GFRT on MHV A59 virus is not at the receptor binding stage, but at the fusion event following the receptor binding.

Conclusion

Lectins are carbohydrate binding agents (CBA) that are being investigated for their anti-viral properties against enveloped viruses [74], and in recent studies, have been shown to be effective against various coronaviruses and other members of the order Nidovirales[79]. Lectins like GFRT can act by binding with glycans on virus surface proteins and preventing the virus-cell interactions required for viral entry[80]. This study revealed that though GFRT does not prevent interaction of MHV A59 spike with its receptor CEACAM, it inhibits cell fusion by up to ~60% . Thus, the inhibition of coronavirus entry by GFRT is at the level of post-receptor binding events. MHV A59 spike is a heavily glycosylated protein with 18 potential N-glycosylation sites. Figure 19 is a schematic depiction of MHV A59 spike with the putative N-glycosylation sites indicated.

Figure 19. Schematic depiction of the putative N-linked glycosylation sites on MHV A59 Spike.

It is possible that GFRT inhibits the fusion events which follow the conformational changes of MHV Spike that are associated with receptor binding[26]. By binding glycans near the fusion peptide, GFRT could obstruct insertion of the fusion peptide into the target cell membrane. It is also possible that GFRT binds to glycans in the vicinity of HR1 and HR2 peptides preventing the formation of the stable six-helix bundle (6HB) which draws the viral and cellular membranes close together for fusion to occur. Further studies of MHV testing the appearance of 6HB in the presence of GFRT may reveal more insight into its site of action.
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VITA

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