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Glycosylation of Mouse DPP4 Plays a Role in Inhibiting Middle East Respiratory Syndrome Coronavirus Infection

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Middle East respiratory syndrome coronavirus (MERS-CoV) utilizes dipeptidyl peptidase 4 (DPP4) as an entry receptor. Mouse DPP4 (mDPP4) does not support MERS-CoV entry; however, changes at positions 288 and 330 can confer permissivity. Position 330 changes the charge and glycosylation state of mDPP4. We show that glycosylation is a major factor impacting DPP4 receptor function. These results provide insight into DPP4 species-specific differences impacting MERS-CoV host range and may inform MERS-CoV mouse model development.

Coronaviruses are a diverse family of single-stranded, positive-sense RNA viruses that have frequently undergone host range expansion events. While coronaviruses have expanded their host range into humans multiple times over the course of their evolutionary history, two recent events have resulted in the emergence of highly pathogenic epidemic strains. First, severe acute respiratory syndrome coronavirus (SARS-CoV) emerged into the human population in 2003 and infected over 8,000 people before finally being contained by aggressive public health intervention strategies. More recently in 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) emerged from its zoonotic host species into humans, resulting in severe disease and a 38% mortality rate. MERS-CoV likely originated from a bat reservoir species, as evidenced by the identification of closely related MERS-CoV-like viruses in bats (1, 2), although current hypotheses suggest that a camel intermediate host also played an important role in the host range expansion event.

The functional receptor for MERS-CoV was recently identified as dipeptidyl peptidase 4 (DPP4) (3). Interestingly, while MERS-CoV can utilize human, bat, and camel DPP4 (data not shown), traditional small animal models are non-permissive, including mice (4, 5), ferrets (6), and hamsters (7). The relevance of MERS-CoV as an emerging pathogen and the importance of

small animal models for studying pathogenesis and for developing vaccines and therapeutics led us to identify the determinants of interactions between the MERS-CoV receptor binding domain (RBD) and mouse DPP4 (mDPP4). Interactions between DPP4 and the MERS-CoV RBD are primarily restricted to blades IV and V of the DPP4 N-terminal β-propeller domain (8, 9). Recently, we found that two key residues in mDPP4 (A288L and T330R) could permit infection by MERS-CoV when mutated to the human DPP4 (hDPP4) amino acids (4). These residues lie within blades IV and V of the β-propeller domain (see 8, 9). The importance of A288L can be understood by recognizing that there is a strong hydrophobic region in the MERS-CoV RBD that engages the equivalent hDPP4 residue (L294) (9). In fact, all permissive DPP4 orthologs have a leucine residue at this site (i.e. bat, camel, human, marmoset). This interaction, however, is altered in mDPP4, potentially making this hydrophobic region less amenable to interacting with the MERS-CoV RBD.

On blade IV, the T330R substitution in mDPP4 regulates two potentially critical virus-host cell receptor interaction events. *First*, the 330 arginine provides a highly conserved charge that is present in all known permissive hosts, but missing from all known non-permissive hosts (Fig. 1A). In hDPP4, the interaction between this residue (R336 relative to hDPP4 numbering) and the MERS-CoV RBD Y499 has been previously noted as a key interaction (8, 9). The absence of this interaction could be a primary factor behind the lack of permissivity of mDPP4, as well as other non-permissive DPP4 orthologs. *Second*, the T330R mutation knocks out an NXT glycosylation motif in mDPP4. Western Blot analysis is consistent with the loss of glycosylation at this site, as evidenced by a ~2.5 kDa downward shift in the mDPP4 T330R mutant (Fig. 1B). Considering these two potentially important effects, we hypothesized that

either the introduction of the conserved charge or the removal of glycosylation was crucial for regulating mDPP4 permissivity to MERS-CoV infection.

To test the impact of glycosylation versus charge on the ability of mDPP4 to support infection by MERS-CoV, we generated a panel of DPP4 mutants (Fig. 1C, 1D) contained within the 945ΔRRE expression vector, a lentiviral vector derived from pTK945. DPP4 constructs were expressed in human embryonic kidney 293T (HEK 293T) cells that lack detectable expression of endogenous hDPP4 (10). At 18 hours post-transfection with 3 µg of the DPP4 expression plasmid, cells were infected with rMERS-CoV-RFP which encodes tomato red fluorescent protein in place of ORF5 (11). Cells were imaged ~24 hours post-infection to assess the number of positive cells as a readout for MERS-CoV infection.

A set of hDPP4 mutants were generated and assayed for permissivity to MERS-CoV infection in order to first assess the importance of glycosylation versus charge in the human context. We generated two mutants: one that included a glycosylation site and one that removed the charge. First, we swapped the three residues of the NLT mDPP4 putative glycosylation site with residues 334 to 336 of hDPP4 (hDPP4 + gly). This addition shows a severe reduction in infection (Fig. 2A, 2B), with an upward shift in the Western Blot band consistent with successful introduction of the glycosylation site (Fig. 2C). However, this mutation impacts both the glycosylation site and the charged 336 residue (aligning to residue 330 in mDPP4, Fig. 1A). Therefore, our second mutant introduces the R336T mutation by itself, which removes the positive charge without introducing glycosylation. While we do observe a decrease in infection, it is not comparable in magnitude to the decrease seen when glycosylation is included (Fig. 2A, 2B), suggesting that the presence of a positively charged residue at position 336 is not essential for hDPP4-mediated MERS-CoV infection. Additionally, the presence of glycosylation does not

93 impact the ability of hDPP4 + gly to be expressed on the cell surface (Fig. 4). These results show
94 that glycosylation can act to inhibit infection by MERS-CoV and that the positive charge is not a
95 crucial interaction in the context of hDPP4.

96 In order to directly assess the relative contribution of charge versus glycosylation in the
97 context of mDPP4, we evaluated whether the presence of glycosylation or charge at the 330 site
98 regulates mDPP4 receptor activity. For these studies, mutations were evaluated singly and in the
99 presence of the secondary mutation (A288L), which is essential for high levels of MERS-CoV
100 receptor activity. Importantly, introduction of the charged residue at 330 simultaneously destroys
101 the glycosylation site, preventing us from testing whether the presence of the charged residue at
102 330 can enhance mDPP4 receptor activity in the presence of a glycosylation site. However, it is
103 possible to remove the glycosylation site without introducing a charged residue with the
104 mutation N328A, which disrupts the N of the NXT motif (Fig. 1A, 1D). When we assessed the
105 N328A mutant in the context of the A288L background we observed high levels of infection
106 (Fig. 3A) that are not statistically different from mDPP4 A288L, T330R (Fig. 3B). Both
107 glycosylation knockout mutants have levels that are statistically greater than mDPP4 but
108 statistically less than hDPP4 (Fig 3B). All mutants containing the T330R or N328A mutation
109 show a ~2.5 kDa downward shift in the Western Blot, consistent with the loss of glycosylation
110 (Fig. 3C). Importantly, surface staining for mDPP4 and hDPP4 signifies that all derivatives of
111 the DPP4 receptors are expressed at the cell surface and available to interact with the MERS-
112 CoV RBD (Fig. 4). Together, these results indicate that removal of the glycosylation site, rather
113 than addition of the charged residue at position 330, is responsible for regulating the ability of
114 MERS-CoV to utilize mDPP4 as a functional receptor. The secondary mutation, A288L, also
115 plays an important role in MERS-CoV permissivity due to the fact that high levels of infection

are only seen when the glycosylation mutants are combined with the A288L substitution (Fig 3A, 3B). Together, this suggests that while glycosylation is an important barrier, its removal is not sufficient to permit infection in the absence of the A to L modification at position 288.

The importance of glycosylation in the interactions between coronaviruses and host-cell receptors has previously been recognized. For example, the introduction of a glycosylation site into human aminopeptidase N (APN) prevents human coronavirus 229E from utilizing it as a receptor (12). For MERS-CoV, it is possible that glycosylation can act as a broader determinant of DPP4-mediated host range, since other non-permissive hosts (i.e. ferrets, hamsters) also have a non-conserved glycosylation site in the region of DPP4 that interacts with the MERS-RBD (Fig. 1A). In the context of a small animal model, the presence of the glycosylation site at 330 may sterically hinder multiple interacting residues between the MERS-CoV RBD and mDPP4, complicating the generation of a mouse-adapted strain. Therefore, it may be necessary to partially or fully humanize mDPP4 to achieve *in vivo* MERS-CoV replication. Additionally, the finding that changes in both blades of mDPP4 is crucial for mediating permissivity to MERS-CoV (Fig. 3A) has two major implications. *First*, it may help inform future studies in other non-permissive hosts, particularly since single amino acid changes have not resulted in successful infections (e.g ferrets (data not shown)). *Second,* it suggests that circulating MERS-like coronaviruses cannot expand their host range into mice and possibly other rodent species with just one change. Rather, extensive remodeling of the MERS-CoV RBD is likely required for it to successfully utilize non-permissive DPP4 orthologs as receptors, especially if glycosylation acts to block infection in these alternate species. Presumably, the modifications that would allow the MERS-CoV RBD to utilize mDPP4 and other orthologs would likely attenuate or even ablate its ability to utilize hDPP4. Overall, by understanding the biochemical determinants that mediate

MERS-CoV utilization of DPP4 orthologs, we can begin to characterize the selective pressures leading up to host-range expansion events, with the broader goal of being able to predict future emergences.

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FIGURE LEGENDS

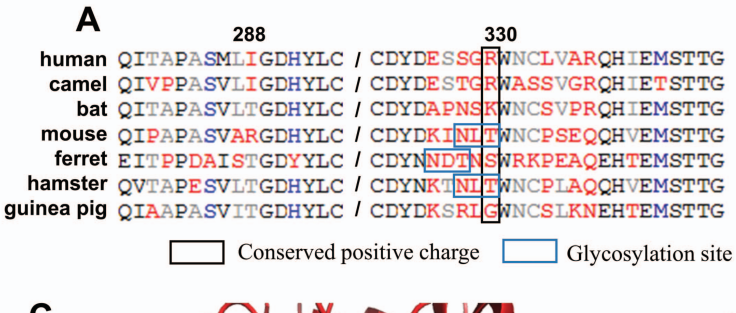
FIG 1. Is charge or glycosylation important for mediating mouse DPP4 permissivity? (A) MEGA6 protein sequence alignment of DPP4 for various permissive (human, camel, bat) and non-permissive (mouse, ferret, hamster, guinea pig) species, visualized in GeneDoc. Residue numbers are relative to mDPP4. The mutation T330R in mDPP4 introduces a conserved positive charge for permissive hosts, but also knocks out a glycosylation site. NCBI accession numbers: human, NP_001926.2; camel, AIG55259; bat, AGF80256.1; mouse, NP_034204.1; ferret, ABC72084.1; hamster, AIG55262.1; guinea pig, XP_003478612.2. (B) The downward shift in the mDPP4 T330R band is consistent with the removal of glycosylation. Western blot protocol follows Cockrell *et al*. 2014. (C) Structure of hDPP4 (yellow) complexed with the MERS-RBD (red) (PDB code 4L72) visualized using PyMOL Molecular Graphics System, Version 1.6.0.0 Schrodinger, LLC. mDPP4 (blue), threaded through i-TASSER (13), is overlaid to show the key mutations: A288L, T330R, N328A. Blue indicates wildtype mDPP4 residues while orange indicates the human amino acid identity. (D) DPP4 constructs used and whether they are glycosylated at the 328 residue or whether the conserved positive arginine is present at the 330 residue (numbered relative to mDPP4).

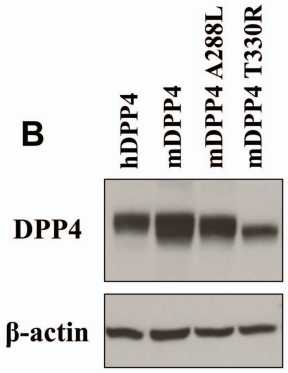
FIG 2. Glycosylation can act to dramatically reduce infection by MERS-CoV. (A) HEK 293T cells were transfected with each DPP4 construct and infected with rMERS-CoV-RFP at an MOI of 1 at ~18 h post-transfection. At ~24 h post-infection, cells were imaged. (B) Cells were transfected as in (A) and infected with rMERS-CoV-RFP at the following MOIs: hDPP4 and hDPP4 R336T, 0.001; no DPP4 and hDPP4 + gly, 0.1. At 24 hours post-infection, cells were counted based on red fluorescence and values were normalized to an MOI of 0.1. Values represent 3 replicates. All mutants have levels that are statistically greater than no DPP4 and all

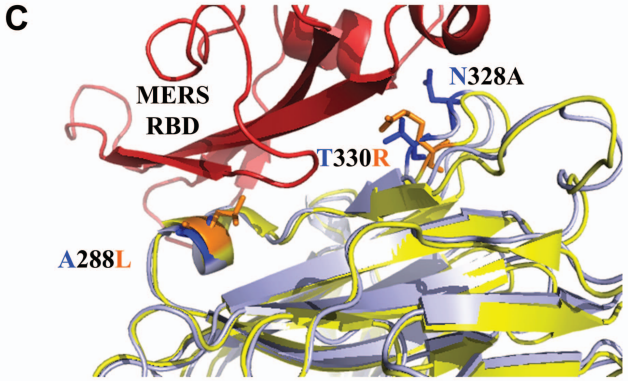
other pairwise comparisons are also statistically significant (indicated by *, $p < 0.05$, Student's t-test). (C) Western blot analysis for MERS nucleocapsid (N) protein, DPP4, and actin as a loading control. Western blot protocol follows Cockrell *et al.* 2014.

FIG 3. Glycosylation, rather than charge, is a key determinant of mouse DPP4 permissivity to MERS-CoV. (A) Cells were transfected and infected following the protocol detailed in Fig 2A. Neither mDPP4 N328A, nor mDPP4 T330R can confer permissivity to MERS-CoV, however both result in strong levels of infection when coupled with A288L. (B) Red cell counts were calculated as in Fig. 2B with the following MOI: hDPP4, 0.001; mDPP4, mDPP4 288, mDPP4 328, mDPP4 330, no DPP4, 0.1; mDPP4 288, 328 and mDPP4 288, 330, 0.01. All DPP4 constructs are significantly greater than no DPP4 and mDPP4 (*,$p < 0.05$, Student's t-test) and significantly less than hDPP4 (+, $p < 0.05$, Student's t-test); however mDPP4 A288L, N328A and mDPP4 A288L, T330R are not statistically different from each other (n.s., $p < 0.05$, Student's t-test). (C) Western blot analysis for MERS nucleocapsid (N) protein, DPP4, and actin as a loading control. Western blot protocol follows Cockrell *et al.* 2014.

FIG 4. DPP4 and mutant variants are expressed on the surface of cells, visible by immunofluorescence. Cells were transfected as described in Fig 2A, fixed, and probed with primary goat-anti-DPP4 polyclonal antibody (R&D Systems) at 1:50 and secondary donkey-anti-goat Alexa Fluor 488 (Life Technologies) at 1:500. Cells were imaged at 40X for DAPI (30 ms exposure) and DPP4 (160 ms exposure).







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Construct	Glycosylated?	Conserved positive charge?
hDPP4	-	X
hDPP4 + gly	X	-
hDPP4 R336T	-	-
mDPP4	X	-
mDPP4 A288L	X	-
mDPP4 N328A	-	-
mDPP4 T330R	-	X
mDPP4 A288L, N328A	-	-
mDPP4 A288L, T330R	-	X