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Identification of residues on human receptor DPP4 critical for MERS-CoV binding and entry

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ABSTRACT
Middle East respiratory syndrome coronavirus (MERS-CoV) infects host cells through binding the receptor binding domain (RBD) on its spike glycoprotein to human receptor dipeptidyl peptidase 4 (hDPP4). Here, we report identification of critical residues on hDPP4 for RBD binding and virus entry through analysis of a panel of hDPP4 mutants. Based on the RBD–hDPP4 crystal structure we reported, the mutated residues were located at the interface between RBD and hDPP4, which potentially changed the polarity, hydrophobic or hydrophilic properties of hDPP4, thereby interfering or disrupting their interaction with RBD. Using surface plasmon resonance (SPR) binding analysis and pseudovirus infection assay, we showed that several residues in hDPP4–RBD binding interface were important on hDPP4–RBD binding and viral entry. These results provide atomic insights into the features of interactions between hDPP4 and MERS-CoV RBD, and also provide potential explanation for cellular and species tropism of MERS-CoV infection.

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Introduction

Middle East respiratory syndrome (MERS), a novel coronavirus which causes severe respiratory illness, was first reported in a patient from Saudi Arabia in 2012 (de Groot et al., 2013). To date, individual cases as well as small clusters and large outbreaks have been reported in several countries and the mortality rate is estimated at 30% among laboratory-confirmed cases (Organization, 2014). Phylogenetic analysis demonstrates that the MERS coronavirus (MERS-CoV) is genetically closest to clade 2c betacoronavirus found in camels and insectivorous bats (Ithete et al., 2013) although the true viral reservoir remains uncertain. The clinical symptoms caused by MERS-CoV are similar to those caused by severe acute respiratory syndrome coronavirus (SARS-CoV) although the two viruses use two distinct receptors; MERS-CoV uses dipeptidyl peptidase 4 (DPP4) while SARS-CoV uses angiotensin-converting enzyme 2 (ACE2). Other coronaviruses use other receptors and perhaps this provides partial explanation for their cellular and species tropism. MERS-CoV can replicate in a range of cell lines derived from human, non-human primate, porcine, and bat (de Wit et al., 2013). Traditional small laboratory animals, such as mice (Coleman et al., 2014), hamsters (de Wit et al., 2013), and ferrets (Raj et al., 2014), were shown to resist MERS-CoV infection. The finite host range of MERS-CoV has seriously restricted the development of appropriate animal models to study the pathogenesis of this virus and to assess the efficacy of potential therapeutic strategies. Raj et al. (2014) demonstrated that human receptor DPP4 (hDPP4) domain (residues 246 to 505) could confer the susceptibility of ferret DPP4 to MERS-CoV infection. Zhao et al. (2014) are the first to describe a method of developing a small-animal model for MERS-CoV in which an adenovirus expressing hDPP4 was utilized to transiently transduce mouse airway cells and make mice susceptible to MERS-CoV infection. Recently van Doremalen et al. (2014) showed that DPP4 played an important role in the observed species tropism of MERS-CoV infection and identified residues in DPP4 responsible for this restriction. These results indicate that the insusceptibility to infection is primarily determined by the inability of MERS-CoV binding to DPP4 of a non-permissive cell line.

Previous findings have shown that hDPP4 extracellular domain consists of a variable N-terminal eight-blade β-propeller domain and a conserved C-terminal αβ-hydrolase domain (Engel et al., 2003; Rasmussen et al., 2003). However, our understanding of critical residues of hDPP4 on MERS-CoV interaction and entry is quite limited. We and others have previously characterized

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RBD–hDPP4 crystal structure ([Chen et al., 2013; Lu et al., 2013; Wang et al., 2013]). The RBD–hDPP4 crystal structure showed that the viral RBD recognized blades IV and V of the DPP4 β-propeller domain. The atomic interaction details of the binding interface were mediated by several amino-acid residue interactions, including RBD residue D539 with hDPP4 residue K267, RBD Y499 with DPP4 R336, RBD residues D510 and E513 with DPP4 residues R317 and Q344, RBD L506, W553 and V555 with DPP4 L294 and I295. Previously, we have generated a panel of MERS-CoV mutant RBD proteins at the residues D539, Y499, D510, E513, L506, W553 and V555 to characterize their impacts on binding activity to hDPP4 and the entry efficiency into target cells. However, the impacts of the corresponding residues on hDPP4 have not been well characterized. Here, through structure-guided mutagenesis, we identified several key residues in hDPP4 that were critical for RBD binding measured by both real-time surface plasmon resonance (SPR) and pseudovirus entry. These residues included K267 and R336 on binding patch 1, and L294, I295, R317 and Q344 on binding patch 2. The mutations of three positively charged residues K267, R336 and R317 perhaps interfere with the interaction of the negatively charged residues on the surface of RBD; the mutations of L284, I295 and Q344 may lead to the change of hydrophobic or hydrophilic properties of hDPP4 at the interface with RBD.

Results

Critical residues on hDPP4 for binding to MERS-CoV RBD

Our previous findings have shown that the binding interface between hDPP4 and MERS-CoV RBD is mainly composed of two binding patches, patch 1 and patch 2 (Fig. 1A). The patch 1 interface is characterized by interactions between C-terminal end of the long linker connecting the RBD β6/β7 strands and the hDPP4 blade 4. The contact in patch 1 is critically determined by the polar interactions among a group of hydrophilic amino-acid residues, including RBD E536, D537, D539 and Y499 and hDPP4 K267 and R336. In this patch, DPP4 residue K267 interacts with RBD D539 by salt bridge (Fig. 1B), while DPP4 residue R336 forms hydrogen bond with RBD residue Y499 (Fig. 1C). Patch 2 has a hydrophobic core surrounded by a hydrophilic periphery. In the hydrophobic core, RBD and hDPP4 contacts are critically dependent on a few ‘hot spot’ residues including RBD L506, W553 and V555, and DPP4 L294 and I295. However, the surrounding hydrophilic surface consists of RBD residues D510, E513 and Y540, and DPP4 residues H298, R317 and Q344. Among these hydrophilic residues, the salt bridge and hydrogen bond between D510 and R317, E513 and Q344 contribute to the maintenance of RBD-receptor contact (Fig. 1D).

To study the impacts of the substitutions of the critical residues on hDPP4 described above on the interaction between MERS-CoV RBD and hDPP4, we determined the binding efficiency between these two proteins by employing SPR technique. First, we constructed a series of hDPP4 mutants guided by the RBD–hDPP4 complex crystal structure information (Wang et al., 2013). The wide-type and mutant hDPP4 were introduced into baculovirus expression system. All wide-type and mutant forms of hDPP4 were expressed efficiently (data not shown). Second, the binding efficiency was measured by SPR. As shown in Fig. 2 and Table 1, mutations at several hDPP4 residues, in individual or combination, resulted in a significant attenuation in binding to MERS-CoV RBD. In patch 1, residue K267 mutation (K267A and K267E) presumably damaged the salt-bridge interaction, completely abrogated the binding between hDPP4 and RBD, while R336A reduced RBD and hDPP4 binding about 100 fold. In patch 2, double mutations at L294 and I295 (L294A+I295A and L294D+I295D) completely eliminated the binding between RBD and hDPP4, presumably by disrupting hydrophobic interactions with RBD L506, W553 and V555. In contrast, the single-residue substitution of R317A and Q344A in the hydrophilic surface of patch 2 had negligible effect on binding efficiency.

Key residues on hDPP4 for MERS-CoV pseudovirus entry into target cells

To further study the importance of the critical residues on hDPP4 on viral entry, we measured the entry efficiency of pseudovirus into COS7 cells expressing the wide-type and mutant forms of hDPP4. The expression levels of the wide-type and mutant hDPP4 were analyzed by fluorescence-activated cell sorting (FACS) using goat anti-hDPP4 polyclonal antibody. All of the wide-type and mutant hDPP4 proteins could be expressed on the surface of COS7 cells with the similar expression efficiency (Fig. 3A). Forty-eight hours later, these cells were exposed to pseudovirus infection and their entry efficiency was measured by luciferase activity 48 h later. As showed in Fig. 3B, the residue mutations located at patch 1 (K267A, K267E and R336A) and hydrophobic region of patch 2 (L294A+I295A and L294A+I295D)
resulted in significantly reduction in viral entry. This is consistent with the binding results described previously. In the hydrophilic region of patch 2, residue substitution R317 led to partial loss of viral infection (41.4%), while the mutation Q344 modestly increased viral infection (22.8%).

**Discussion**

In summary, we have identified several key residues in hDPP4 critical for viral binding and entry into target cells. These residues include positively charged residues of patch 1 (K267 and R336) and hydrophobic zone of patch 2 (L294 and I295). In contrast, the mutations at hydrophilic zone of patch 2 (R317 and Q344) had little influence on binding and virus entry efficiency. These results showed that the positively charged residues at the outer surface of blade 4 and the hydrophobic regions of blade 5 may play an important role in mediating viral binding and entry into the target cells, while the impact of mutations at hydrophilic region of patch 2 was barely detectable. This is consistent with our earlier findings (Wang et al., 2013) where residue mutations at the corresponding negatively charged and hydrophobic core positions on RBD of MERS-CoV could significantly reduce both binding and viral entry efficiency.

Sequence analysis of DPP4 from multiple animal species (Fig. 4) showed that MERS-CoV susceptible animals, such as macaque, camel and bat, shared the same sequence with hDPP4 at blades IV and V. In contrast, those MERS-CoV resistant animals, such as mouse, rat and ferret, have residues at L294, I295 and R366 that are all different from hDPP4. Raj et al. (2014) reported that when these sites of hDPP4 were changed to the residues of ferret, the binding and viral infection efficiency could also be decreased. van Doremalen et al. (2014) found 5 residues involved in the hDPP4–RBD interaction which were important to determine the susceptibility to MERS-CoV infection, in which I295 and R336 were included. These results are consistent with our findings and suggest these residues play an important role in RBD binding and viral entry, and determining the tropism to MERS-CoV infection.

**Materials and methods**

Constructs and protein expression

MERS-CoV RBD (residues 367-606) and the extracellular domain of hDPP4 (residues 39-766) were expressed using a Bac-to-Bac® baculovirus expression system (Invitrogen). In brief, the DNA encoding RBD and hDPP4 were respectively cloned into the pFastBac™ dual vector (Invitrogen) incorporating an N-terminal gp67 signal peptide to facilitate secretion and a C-terminal hexa histidine-tag for purification. The constructed DNA was then transformed into the bacterial DH110Bac competent cells and the recombinant bacmid DNA was extracted and transfected into Sf9 cells using Cellfectin II Reagent (Invitrogen). After 5–7 days of incubation at 300 K, the low-titer viruses were harvested and then amplified. The amplified high-titer viruses were then used to infect Sf9 cells and the cell culture supernatant containing target protein was harvested 60 h after infection, concentrated, loaded to nickel (Ni)-charged resin (GE Healthcare), and eluted with 0.5 M imidazole and further purified using the Superdex™ 200 high-performance column (GE Healthcare) pre-equilibrated with Tris...
buffer (50 mM Tris, pH 8.8, 40 mM NaCl). Fractions containing the purified protein were collected and applied directly to a pre-equilibrated Resource™ Q column (GE Healthcare) and then eluted with a 0.05–1 M NaCl gradient in 40 mM Tris buffer (pH 8.8). Fractions containing protein were finally purified using Superdex™ 200 column pre-equilibrated with HBS (10 mM HEPES, pH 7.2, 150 mM NaCl) and centrifuged to 1 mg/ml. Mutants of the extracellular domain of hDPP4 were constructed using a standard PCR-based cloning strategy. And the mutant proteins were expressed and purified in the same way.

**SPR analysis**

The SPR analyses were carried out using a Biacore T200 instrument (GE Healthcare) equipped with a research-grade CM5 sensor chip. To measure the affinity binding between RBD and wild-type or mutant hDPP4, the RBD was immobilized on the sensor chip by standard amine coupling procedure. The flow cell 1 was left blank to serve as a reference. Purified RBD at a concentration of 5 μg/ml in sodium acetate buffer (10 mM, pH 5.0) was immobilized to a density of 300–400 response units on the flow cell 2. For the collection of binding data, hDPP4 or its mutants in a buffer of 10 mM HEPES, pH 7.2, 150 mM NaCl, and 0.005% (v/v) Tween-20 were injected over the two flow cells at a series of concentration at a 30 μl/min flow rate and 298 K. The RBD–hDPP4 complex was allowed to associate for 60 s and dissociated for 60 s. The surfaces were regenerated with an injection of 5 mM NaOH between each cycle if needed. The data was analyzed with the Biacore T200 evaluation software by fitting to a 1:1 Langmuir binding model.

**MERS-CoV pseudovirus and viral infection**

MERS-CoV pseudovirus was generated by co-transfection of human immunodeficiency virus (HIV) backbone expressing firefly luciferase (pNL43R-E-luciferase) and MERS-CoV spike glycoprotein expression vector (pcDNA3.1⁺, Invitrogen) into the 293 T cells. Viral supernatants were harvested 48 h later, normalized by p24 ELISA kit (Beijing Quantobio Biotechnology Co., LTD, China) before infecting the target COS7 cells transiently expressing wild-type or mutant hDPP4. The wide-type and mutant hDPP4 expressing COS7 cells were incubated with goat anti-hDPP4 polyclonal antibody (R&D) followed by incubation with fluorescein phycoerythrin (PE)-labeled rabbit anti-goat IgG antibody (Santa Cruz). The expression levels of wide-type and mutant hDPP4 were measured by flow cytometer (BD Aria II) and the mean fluorescence intensity (MFI) was analyzed. The COS7 cells infected by MERS-CoV pseudovirus were lysed at 48 h post infection and viral entry efficiency was quantified by comparing the luciferase activity between pseudoviruses-infected COS7 cells expressing wide-type and those infected COS7 cells expressing mutant hDPP4.

**Fig. 3.** Infection efficiency of MERS-CoV pseudoviruses into COS7 cells expressing wide-type or mutant hDPP4. (A) hDPP4 expression on COS7 cells. COS7 cells transiently transfected with different hDPP4 constructs were used as target cells for pseudovirus infection. The mean fluorescence intensity (MFI) of target cells and control cells (without hDPP4 transfection) incubated with fluorescent antibody was determined by flow cytometer. The result shown is representative of three independent experiments conducted in triplicate. The actual residue mutants in hDPP4 are indicated below the horizontal axis. (B) The entry efficiency (%) of pseudovirus was calculated on the basis of luciferase activity. And the percentages of pseudovirus entry efficiency shown for mutant hDPP4 were luciferase activity values versus that of the wide-type hDPP4, as the entry efficiency for wide-type hDPP4 was defined as 100%. Data shown were corrected for the expression of different hDPP4 constructs by the parameter of MFI. Error bars represent standard errors of the means of three independent experiments. Student’s t-test; *P < 0.05; **P < 0.01.

**Fig. 4.** Amino acid sequence alignment of DPP4 blades IV and V from different species. The mutated positions are highlighted in red boxes.
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