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Peptide Sequence Region That is Essential for the Interactions of the Enterotoxigenic Bacteroides fragilis Metalloproteinase II with E-cadherin

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Abstract

Bacteroides fragilis is a valuable anaerobic commensal and an essential component of the gut microbiome in humans. The presence of a short pathogenicity island in the genome is predominantly associated with the enterotoxigenic strains of B. *fragilis*. Metalloproteinase II (MPII) and fragilysin (FRA) are the structurally related enzymes encoded by the pathogenicity island in the enterotoxigenic strains. Accordingly, there is a significant overlap between the cleavage preferences of MPII and FRA. These proteinases, however, are counter-transcribed in the bacterial genome suggesting their distinct and specialized functions in the course of infection. It is well established that FRA directly cleaves E-cadherin, a key protein of the cell-to-cell adhesion junctions in the intestinal epithelium. Counterintuitively, MPII directly binds to, rather than cleaves, E-cadherin. Structural modeling suggested that a potential E-cadherin binding site involves the C-terminal -helical region of the MPII catalytic domain. The sequence of this region is different in MPII and FRA. Here, we employed substitution mutagenesis of this C-terminal -helical region to isolate the MPII mutants with the potentially inactivated E-cadherin binding site. Overall, as a result of our modeling, mutagenesis and binding studies, we determined that the C-terminal ten residue segment is essential for the binding of MPII, but not of FRA3, to E-cadherin, and that the resulting MPII•E-cadherin complex does not impair E-cadherin-dependent cell-to-cell contacts. It is possible to envision that the putative cleavage targets of MPII should be explored not only on the host cell surface but also in B. *fragilis*.

Keywords: *Bacteroides fragilis*, E-cadherin, Cell receptor, Virulence, Proteolysis, Fragilysin, Metalloproteinase II.

1. Introduction

The role of infectious and inflammatory processes in colon inflammatory diseases and colon carcinogenesis is of great interest. In humans, the largest concentration of microbes is found in the colon. The gram-negative, anaerobic *Bacteroides* is one of the most prominent genera of the human intestinal microbiome. Commensal B. *fragilis* strains are critical to systemic and mucosal immunity and host nutrition [1]. The presence of a single, 6 kb long, pathogenicity island in B. *fragilis* strains in linked to their enterotoxigenicity. Enterotoxigenic, virulent B. *fragilis* strains are involved in multiple chronic inflammatory diseases in the gut in humans and they accelerate the colorectal cancer onset in mice models

[2–4]. There is a consensus that two secretory metalloproteinases, metalloproteinase II (MPII) and fragilysin (FRA) are virulence factors in B. *fragilis* [5–8]. FRAs exist in three highly homologous isoforms (FRA1, 2 and 3), while the sequence identity between FRAs and MPII is only 25%. The crystal structure of FRA3 was reported in 2011 [9]. Recently, we solved the crystal structure of MPII [10]. Despite the low sequence identity between MPII and FRA3, the three-dimensional structures are related. They comprise a large, N-terminal 32-184 regulatory domain unrelated to any known folds, followed by a C-terminal 217-396 catalytic domain connected by a 185-216 linker. The fold of the catalytic domain of both MPII and FRA is largely conserved in metalloproteinases from the archaea kingdom to bacteria and eukaryotes and shows the classical metzincin architecture with the catalytic Zn ion at the bottom of the active site cleft [11].

Importantly, MPII and FRA are counter-transcribed in the bacterial genome [12]. This transcriptional regulation suggests that regardless of their structural similarity and overlapping cleavage preferences these proteinases perform distinct and specialized functions in the course of B. *fragilis* infection. In our attempts to elucidate the functional role of MPII in pathogenesis, we determined that the catalytic domain of MPII directly binds to, but does not cleave, E-cadherin that is a main component of the cell-cell adhesion junctions and an abundant cell surface protein in the intestinal epithelium [32]. In contrast, FRA directly cleaves, rather than binds to, E-cadherin [13, 14]. Because E-cadherin plays a principal role in maintaining normal epithelial cell morphology, its diversified interactions with these two B. *fragilis* proteinases warranted an additional investigation.

Here, we used structure-based mutagenesis followed by the modeling and binding studies of MPII to identify the unique sequence region that is directly involved in the interactions of MPII with E-cadherin. As a result, we determined that (i) the C-terminal ten residue segment that has a low homology level between MPII and FRA is essential for the binding of MPII, but not of FRA3, to E-cadherin, and (ii) the MPII binding does not impair E-cadherin homodimer formation and cell-to-cell contacts [15].

2. Materials and Methods

2.1 General reagents and antibodies

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. McCoy's 5A cell culture growth medium, sulfosuccinimidyl-2-(biotin-amido) ethyl-1,3-dithiopropionate (EZ-Link sulfo-NHS-SS-biotin) and a SuperSignal West Dura Extended Duration Substrate kit were from Thermo Fisher Scientific (Waltham, MA). A TMB/M substrate and the horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgGs were from EMD Millipore (Temecula, CA) and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively.

2.2 Cells

Human colorectal carcinoma HT29 cells were originally obtained from ATTC (Manassas, VA). Cells were routinely grown in the McCoy's 5A medium supplemented with 10 % fetal bovine serum (FBS) and gentamicin (10 μg/ml).

2.3 Cloning, expression and purification of the MPII and FRA3 wild-type and mutant constructs

Cloning of the wild type MPII and FRA3 proenzymes and the MPII-E352A and FRA3-E349A proteolytically inactive mutants, in which Ala replaced the catalytically essential Glu residue of the active site, were described previously [16]. The MPII-E352A-QK, MPII-E352A-DN, MPII-E352A-RMEQK and MPII-E352A- mutants tagged with the N-terminal 6xHis and Flag tags were obtained by mutagenesis of the C-terminal 385-396 Asp-Asn-Trp-Val-Arg-Met-Leu-Glu-Cys-Trp-Gln-Lys-COOH region of MPII. These mutants were constructed using the PCR mutagenesis with MPII-E352A construct as a template and the 5′−TCACCCTCCGATGCACTCCAGCATGCGCACCCAGTTATCGGCGCTCAGGTGG TTAGGGGTAACCTG−3′, 5′−TCACTTTTGGATGCACTCCAGCATGCGCACCCAGC CACCGGCGCTCAGGTGGTTAGGGGTAACCTG −3′, 5′− TCACCCTCCGATGCACC CCAGCCCGCCCACCCAGTTATCGGCGCTCAGGTGGTTAGGGGTAACCTG −3′ and 5′−TCAGGCGCTCAGGTGGTTAGGGGTAACCTG −3′ oligonucleotides as the reverse primers, respectively (mutant nucleotides are underlined). The 5′−CACCATGCACCATC ACCATCACCATGGAGACTACAAAGATGACGATGACAAGGCCTGTGCCGATGACC TG−3′ forward primer was common for all four MPII constructs. Similarly, the original FRA3-E349A construct as a template, and the 5′−CACCATGCACCATCACCATCACCAT GGAGCCTGCAGCAATGAGGCC−3′ and 5′−TCAGCCCAGGTTCTTGGCGATGATG TC−3′ nucleotides as the forward and reverse primers, respectively, were used to generate the FRA3-E349A-Δ construct. The authenticity of the constructs was confirmed by DNA sequencing.

The recombinant pET101 plasmids were used to transform competent E.*coli* BL21 (DE3) Codon Plus cells (Stratagene, La Jolla, CA). Transformed cells were grown at 30°C in LB broth containing ampicillin (0.1 mg/ml). Cultures were induced with 0.6 mM isopropyl β-D-1-thiogalactopyranoside for 16 h at 30°C. Cells were collected by centrifugation, re-suspended in Tris-HCl buffer, pH 8.0, containing 1 M NaCl, and disrupted by sonication. The pellet was removed by centrifugation ($40,000\times$g; 30 min). The constructs were then purified from the supernatant fraction on a Co^{2+}-chelating Sepharose Fast Flow column (GE Healthcare, Little Chalfont, Buckinghamshire, UK), equilibrated with 20 mM Tris-HCl buffer, pH 8.0, supplemented with 1 M NaCl. After washing out the impurities using the same buffer supplemented with 40 mM imidazole, the bound material was eluted using a 40-500 mM gradient of imidazole. The fractions containing the recombinant proteins were combined and then dialyzed against 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl. The purified material was kept at -80°C until use. The purity of the material was tested by SDS gel-electrophoresis (12% NuPAGE-MOPS; Life Technologies, Grand Island, NY) followed by Coomassie staining and by Western blotting with an anti-Flag antibody (Fig.1).

2.4 Biotinylation of the MPII and FRA3 constructs

The purified MPII-E352A, MPII-E352A-QK, MPII-E352A-DN and FRA3-E349A recombinant constructs (50 μl at 2 mg/ml each) were labeled for 30 min on ice at a 1 : 20 protein-biotin molar ratio using EZ-Link sulfo-NHS-SS-biotin. Excess biotin was removed using a desalting spin-column (Thermo Fisher Scientific, Waltham, MA).

2.5 Binding of MPII and FRA3 to the cells

HT29 cells were grown in wells of a 12-well plate in McCoys 5A medium-10% FBS until a 80-90% confluence level was reached. Cells were then washed with PBS and co-incubated for 3 h at 37C with biotin-labeled MPII-E352A (b-MPII-E352A, 1-10 μg/ml), MPII-E352A-QK (b-MPII-E352A-QK, 1-10 μg/ml), MPII-E352A-DN (b-MPII-E352A-DN, 1-10 μg/ml) or FRA3-E349A (b-FRA3-E349A, 10 μg/ml) in 0.7 ml of serum-free medium. Cells were next extensively washed with ice-cold PBS, and lysed for 1 h at 4°C using 50 mM OG buffer [50 mM octyl--D-glucopyranoside in TBS, pH 7.4, supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 10 μM GM6001 and a proteinase inhibitor cocktail (100 mM AEBSF, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin and 1.5 mM pepstatin A)]. Insoluble material was removed by centrifugation (14,000× g; 15 min). The supernatant aliquots (10 μg total protein) were separated by non-reducing SDS-gel electrophoresis in a 4-12% gradient NuPAGE-MOPS gel and analyzed by Western blotting with either HRP-conjugated ExtrAvidin (dilution 1 : 10,000) or the Hisx6-tag antibody (dilution 1 : 21,000) followed by the HRP-conjugated species-specific secondary antibody (dilution 1 : 5,000) and either a SuperSignal West Dura Extended Duration Substrate kit or a TMB/M substrate.

2.6 Immunoprecipitation of E-cadherin and MPII-E352A

HT29 cells were grown in a 150 mm diameter tissue culture dish in McCoy's 5A medium-10% FBS to reach a 75-80% confluence level. Cells were then washed with ice-cold PBS and lysed for 1 h at 4°C using 50 mM OG buffer. Insoluble material was removed by centrifugation (14,000× g; 15 min). To reduce the detergent concentration, the resulting cell extract was then diluted 2.5-fold in TBS, pH 7.4. Extract aliquots (2 mg total protein in 1.5 ml of 20 mM OG buffer each) were pre-cleared for 2 h at 4°C using the anti-FLAG M2 affinity gel (50 μl, 50% slurry). Following centrifugation to remove the anti-FLAG M2 beads, the samples were either left intact or co-incubated for 2 h at 4°C with MPII-E352A-QK or MPII-E352A-DN (3 μg each). MPII-E352A-QK and MPII-E352A-DN were then pulled-down for 16-18 h at 4°C using the anti-FLAG M2 affinity gel (50 μl, 50% slurry). Following extensive washes in 20 mM OG buffer followed by two washes in TBS, the captured material was eluted from the beads using the FLAG peptide (0.3 mg/ml, 30 μl, 1 h, 4°C). The eluted samples were then separated by SDS-gel electrophoresis in a 4-12% gradient NuPAGE-MOPS gel and analyzed by Western blotting with the monoclonal His6x and E-cadherin antibodies (dilution 1 : 1,000 and 1 : 3,000, respectively) followed by the secondary HRP-conjugated donkey anti-mouse antibody (dilution 1 : 5,000) and a SuperSignal West Dura Extended Duration Substrate kit or a TMB/M substrate.

In addition, HT29 cells (2.5×10^7) were washed with PBS and either left intact or co-incubated for 90 min at 37°C with MPII-E352A-QK or MPII-E352A-DN (1 μg/ml each) in 25 ml of serum-free McCoys 5A medium. Cells were next extensively washed with ice-cold PBS, and lysed for 1 h at 4°C using 50 mM OG buffer. Insoluble material was discarded by centrifugation (14,000× g; 15 min). The cell extracts (2 mg total protein in 30 mM OG buffer) were then incubated for 16-18 h at 4°C with the anti-FLAG M2 affinity gel (50 μl, 50% slurry). Following extensive washes in 30 mM OG buffer, followed by 2 washes in TBS, the bound material was eluted using the FLAG peptide and analyzed by Western blotting as described above.

2.7 Slow E-cadherin-dependent cell aggregation assay

Assays were conducted in wells of a 96-well culture plate coated with 0.05 ml semi-solid agar medium consisting of 100 mg agar (BactoAgar, BD Biosciences, Franklin Lakes, NJ) in 15 ml sterile Ringers salt solution (147 mM NaCl, 4 mM KCl, 2.2 mM $CaCl_2$ and 2.8 mM $NaHCO_3$, pH 7.4). After incubation at 4°C for 1 h to solidify the agar, a single-cell suspension (2×10^4/well) was seeded onto the agar. Cells were either left intact or co-incubated for 24 h with MPII-E352A-QK (10 μg/ml), MPII-E352A-DN (10 μg/ml), FRA3-E349A (10 μg/ml), FRA3 (500 ng/ml) or EGTA (3 mM). Cell aggregates were then evaluated using a bright-field microscope equipped with MagmaFire digital camera.

3. Results

Our previous studies demonstrated that the binding to the cellular E-cadherin occurs via the catalytic domain of MPII and that the presence of the prodomain in the MPII constructs is not essential for the MPII-E-cadherin interactions [32]. Similarly to the X-ray data of the FRA3 crystals, the MII crystals were also organized by protein homodimers [9, 10]. According to the immunoblotting analysis of the purified MPII-E352A samples, the catalytic domain of MPII was represented in solution by roughly equivalent amounts of its monomeric and homodimeric forms (Fig. 1). Our additional experiments demonstrated that the catalytic domain monomers, but not the dimers, were capable of associating with colon carcinoma HT29 cells that expressed high levels of E-cadherin [14, 17]. The bound MPII forms were then readily detectable by Western blotting of the HT29 cell lysate samples (Fig. 1). These results, especially if combined, suggested that the E-cadherin binding site was hidden in the MPII dimers and, accordingly, that the dimerization interface overlapped, at least, partially with the E-cadherin binding site in the MPII monomer structure.

In the PDB 4ON1 structure of the full-length MPII proenzyme the extensive dimerization interface included both the sequences of the prodomain and the catalytic domain. However, in the MPII catalytic domain alone, the short dimerization interface included the C-terminal 385-396 Asp-Asn-Trp-Val-Arg-Met-Leu-Glu-Cys-Trp-Gln-Lys-COOH region. This region is largely represented by the α7 helix in the PDB 4ON1 structure. The earlier studies by other [8, 18] suggested that the C-terminal region plays an important role in the regulation of the functionality of FRA that is structurally similar with MPII. The C-terminal region sequence, however, is divergent in MPII and FRA. In addition, the 397-402 Glu-Ile-Ala-Asp-Gly-Asp-COOH segment (residues are numbered according to the MPII numbering) that is present in FRA is lacking in the peptide sequence of MPII. Accordingly, in our attempt to identify the E-cadherin binding site in MPII, our mutagenesis studies specifically targeted the C-terminal region of the MPII and FRA3 catalytic domains (Fig. 1).

Deletion of the seven residue long Trp-Glu-Ile-Ala-Asp-Gly-Asp-COOH 396-402 C-terminal segment followed by the expression of the recombinant construct in E. *coli*, resulted, however, in the aberrantly folded, insoluble FRA-E349A-Δ mutant. Similarly, deletion of the entire 385-396 Asp-Asn-Trp-Val-Arg-Met-Leu-Glu-Cys-Trp-Gln-Lys-COOH segment or extensive Gly substitution mutagenesis of this sequence resulted in the unfolded, insoluble MPII-E352A-Δ and MPII-E352A-RMEQK mutants, respectively. These data correlate well with the earlier observations by others in FRA [18] and indicate an important role of

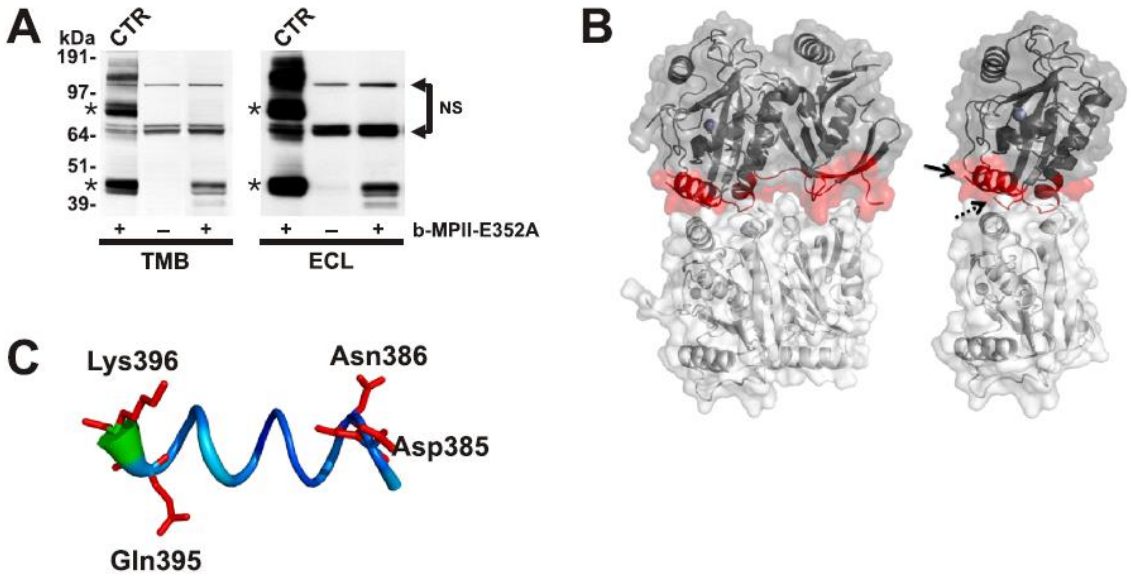

Fig. 1: The MPII catalytic domain monomer binds cellular E-cadherin.
(A) Colon carcinoma HT29 cells were left intact or incubated for 3 h at 37°C with the biotin-labeled purified MPII-E352A sample (b-MPII-E352A; 7.5 μg/ml). Cells were then lysed. Cell lysates were analyzed by Western blotting with ExtrAvidin-HRP followed by a TMB/M substrate (TMB; left) or a SuperSignal West Dura Extended Duration Substrate kit (ECL; right). Control (CTR), b-MPII-E352A (10 ng) was directly added to the HT29 cell extract (total protein, 10 μg). NS, non-specific bands. The monomer and dimer bands of MPII are labeled with an asterisk. These experiments were performed multiple times with the similar results. Representative blots are shown.
(B) Dimerization interface in MPII (PDB 4ON1). Left panel, the MPII proenzyme dimer. The catalytic domain and the prodomain are on the left and right, respectively. The interacting monomers are colored in shades of grey. Right panel, the MPII catalytic domain dimer. The dimerization interface is in red. The solid and dashed lines point to the MPII C-end and to the N-end of the emerging mature proteinase following the removal of the prodomain.
(C) The C-terminal 385-396 α7 helical region of the MPII catalytic domain. The 385-396 Asp-Asn-Trp-Val-Arg-Met-Leu-Glu-Cys-Trp-Gln-Lys-COOH region is shown as a blue/green tube the width of which is proportional to the B-factor (a measure of mobility of a particular part of a protein) that varied from 17.68 (Val388) to 37.25 Å^2 (Lys396). The mutant residues (Asn-Asp in the MPII-E352A-DN mutant and Gln-Lys in the MPII-E352A-QK mutant) are shown as sticks. The final models are displayed using PyMol (www.pymol.org).

the C-terminal sequence of the catalytic domain in the proper folding of both MPII and FRA.

However, less extensive mutagenesis of the MPII C-terminal region such as substitution of the C-terminal 395-396 Gln-Lys and 385-386 Asp-Asn for Gly-Gly followed by the expression of the recombinant constructs in E. coli resulted in the properly folded, soluble full-length MPII-E352A-QK and MPII-E352A-DN mutant proenzymes (Fig. 2).

The purified MPII-E352A-QK and MPII-E352A-DN mutant constructs were then used in the binding experiments in which the biotin-labeled mutants were allowed to associate E-cadherin expressed by HT29 cells. The purified MPII-E352A and FRA3-E349A original constructs were used as controls in our binding experiments. Thus, in agreement with our

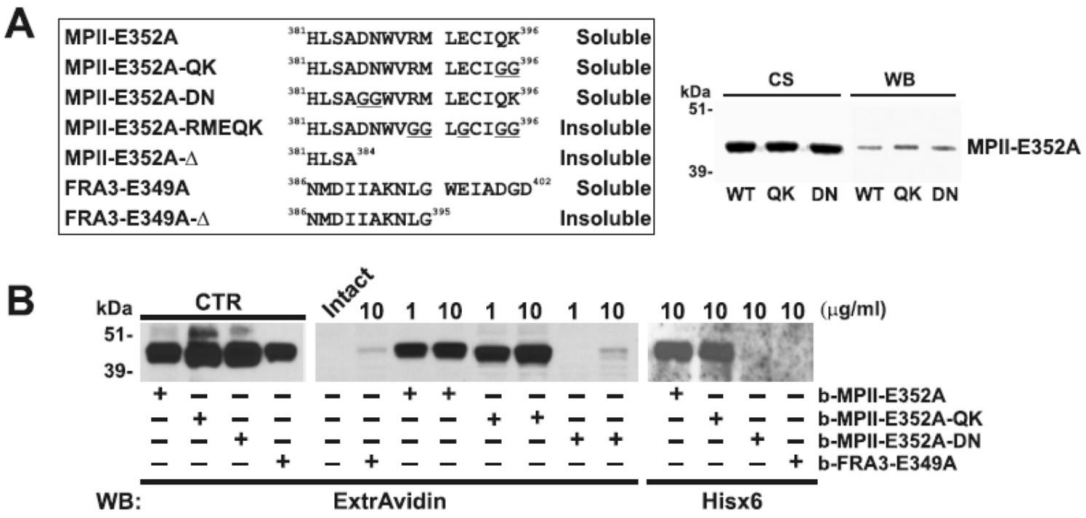

Fig. 2: MPII mutants.
(A) MPII and FRA3 constructs. Left panel, the construct name abbreviations, the C-terminal sequences (residues are numbered according to the MPII numbering) and the solubility/insolubility parameters of the E. *coli*-expressed constructs are shown. Mutant residues are underlined. Right panel, purified MPII constructs. WT, QK and DN, the original MPII-E352A construct, and the MPII-E352A-QK and MPII-E352A-DN mutants, respectively. The purified samples were separated by SDS-gel electrophoresis in 12% NuPAGE-MOPS gels. CS, Coomassie staining (2 g total protein each), WB, Western blotting (100 ng total protein each) with a FLAG antibody.
(B) E-cadherin binding is impaired in the MPII-E352A-DN mutant. HT29 cells were left intact or incubated for 3 h at 37°C with the indicated concentrations of the biotin-labeled MPII and FRA3 constructs (b-MPII-E352A, b-MPII-E352A-QK, b-MPII-E352A-DN and b-FRA3-E349A). Cells were then lysed. Cell lysates were analyzed by Western blotting with ExtrAvidin-HRP (left and middle) and a Hisx6-tag antibody (right). Control (CTR), the b-MPII-E352A, b-MPII-E352A-QK, b-MPII-E352A-DN and b-FRA3-E349A samples (10 ng each) were directly added to the HT29 cell extract (total protein, 10 μg). Representative blots from three independent experiments are shown.

earlier observations MPII, but not FRA3, readily bound cellular E-cadherin in HT29 cells. The MPII-E352A-QK mutant was capable of binding E-cadherin as efficiently as the original MPII constructs. In turn, the MPII-E352A-DN mutant, in which Gly-Gly substituted for the Asp-Asn sequence in the α7 helix, was unable to bind to E-cadherin. As a result, we are now confident that the α7 helix region is essential for the binding of MPII with cellular E-cadherin (Fig. 2).

To further support our data, we co-incubated HT29 cells with the purified MPII-E352A-QK and MPII-E352A-DN constructs tagged with both the FLAG- and His6x-tags. We then lysed the cells and pulled-down, using the FLAG M2-antibody beads, the cell surface-associated MPII•E-cadherin complex from the cell lysate aliquots. The precipitated material was analyzed by Western blotting using the monoclonal E-cadherin and His6x-tag antibodies. Our results indicated that only MPII-E352A-QK, but not MPII-E352A-DN, was pulled-down jointly with cell surface-associated E-cadherin (Fig. 3).

To confirm that the MPII-E352A-DN mutant was incapable of binding to E-cadherin, we lysed HT29 cells and then added the MPII-E352A-QK and MPII-E352A-DN constructs to the lysate. The MPII•E-cadherin complex was pulled-down, using the FLAG M2-antibody beads, from the samples and the isolated material was by Western blotting using the mon-

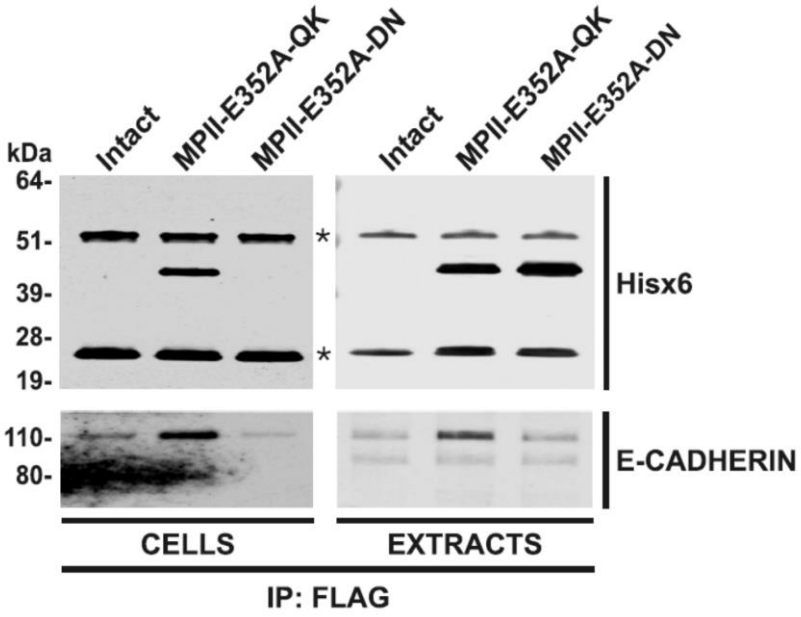

Fig. 3: MPII-E352A-QK mutant, but not the MPII-E352A-QK mutant, interacts with E-cadherin. Left panels, HT29 cells were left intact or co-incubated for 3h at 37°C with the FLAG- and His6x-tagged MPII-E352A-QK or MPII-E352A-DN mutant (1μg/ml each). Cell surface-associated MPII-E352A-QK and MPII-E352A-DN were then immunoprecipitated (IP) from the cell lysates, using the FLAG M2 antibody beads. Right panels, HT29 cell extracts (2mg total protein each) were left intact or co-incubated with the FLAG- and His6x-tagged MPII-E352A-QK or MPII-E352A-DN mutants (3μg each). The MPII-E352A-QK or MPII-E352A-DN constructs were then immunoprecipitated, using the FLAG M2 antibody beads. Captured material was eluted from the beads using the FLAG peptide, and then analyzed by Western blotting with the His6x (top) and the E-cadherin (bottom) antibodies. The heavy and light chains of the FLAG M2 antibody are labeled with an asterisk.

oclonal E-cadherin and His6x-tag antibodies. Again, only the MPII-E352A-QK construct co-precipitated E-cadherin indicating that MPII-E352A-QK, but not the MPII-E352A-DN construct, was capable of binding to cellular E-cadherin (Fig. 3). Taken together, our results clearly demonstrated that the binding of the MPII-E352A-QK construct, but not of the MPII-E352A-DN mutant, to the HT29 cell surfaces occurred through the direct interaction of MPII with E-cadherin.

Because E-cadherin is the major Ca^{2+}-dependent transmembrane protein of the cellular adherens junctions and a key player in cell-to-cell contacts that take place via E-cadherin homophilic dimerization [15], we next determined if the binding of the MPII constructs to E-cadherin interferes with E-cadherin-dependent cell aggregation. Thus, HT29 cells were left intact or co-incubated with the purified MPII-E352A-QK and MPII-E352A-DN constructs and also with active FRA3 that is known to cleave E-cadherin. In addition, we used the catalytically inert FRA3-E349A mutant as a control. As we expected, active FRA3 repressed cell aggregation in a way that was similar with EGTA, a Ca^{2+} ion chelator. In contrast, both the inactive FRA3-E349A mutant and the MPII-E352A-QK and MPII-E352A-DN mutants were inert in this assay and they did not affect the cell aggregation status any significantly as compared with the intact cells (Fig. 4). These observations strongly suggest that the binding of MPII to E-cadherin does not interfere with cell-to-cell adhesion contacts.

4. Discussion

MPII, a potential virulence factor, and FRA (also called BFT or *Bacteroides fragilis* Toxin) are two metalloproteinases encoded by the pathogenicity island in multiple pathogenic B. *fragilis* strains [8,19]. The presence of the pathogenicity island in the genome is linked to enterotoxigenic B. fragilis [20-22]. FRA exists in three highly homologous enterotoxigenic isoforms (FRA1, FRA2, and FRA3), which differ by only a few substitutions [6,22-30]. The MPII and FRA proteinases are secreted by the bacteria as the inert proenzymes that include the N-terminal $\sim$ 150 residue prodomain and the C-terminal catalytic domain of $\sim$ 180 residues connected by a 20 residue long linker. Despite a low, 25%, sequence identity between FRA and MPII, the fold of the catalytic domain in MPII is similar to that in FRA3 [9,10,16]. In contrast to the catalytic domain, the prodomain of MPII exhibits an unconventional fold. This prodomain fold is similar to that of FRA3 but not to any other known proteins [9,10]. In the course of the proenzyme activation in MPII and FRA, the prodomain is cleaved by the external proteinases and, as a result, the mature proteinases of MPII and FRA are liberated.

The main known cleavage function of the FRA proteinase is the proteolysis of E-cadherin, a key component of cell-cell contacts in the epithelium [8,13,31]. By cleaving E-cadherin, FRA is likely to weaken cell-to-cell contacts, enabling enterotoxigenic B. *fragilis* to penetrate the intestinal epithelium and to cause abscesses and inflammation within the tissue. In turn, as we recently demonstrated both the full-length MPII proenzyme and the MPII enzyme were capable of directly binding to E-cadherin in a way that is similar to the ligand-receptor interactions [32]. No MPII proteolysis of E-cadherin was recorded in the course of these binding events. Because there is a significant overlap of the cleavage preferences of FRA *versus* MPII, this unexpected function of MPII warranted further studies.

To shed light on the potential function of MPII, we investigated the MPII-E-cadherin interactions in more detail. In the course of our study, we determined that the MPII catalytic domain monomers were able to bind cellular E-cadherin while the homodimers of MPII were incapable of associating with E-cadherin. These findings suggested that the putative E-cadherin binding site is hidden in the MPII homodimers but it is exposed in the monomers. The follow-on analysis of the PDB 4ON1 crystal structure of MPII we recently reported [10] suggested that the dimerization interface involves the 7 helix of the MPII C-terminal region. In addition, the C-terminal region is divergent in MPII and FRA and, accordingly, may represent the putative E-cadherin binding site in MPII rather than in FRA. As a result, we specifically selected the C-terminal segment of MPII for our mutagenesis studies.

To destroy the helical structure of the C-terminal region and to inactivate its ability to bind to E-cadherin, we used substitution mutagenesis of MPII. As expected, the MPII-E352A-DN mutant, in which Gly-Gly substituted for the Asp-Asn sequence in the 7 helix, was unable to bind to E-cadherin. This finding confirms our suggestion that the C-terminal region of MPII is directly involved in the binding to cellular E-cadherin. In addition, we may conclude that MPII binding site is localized outside of the dimerization interface of E-cadherin that is localized in the E-cadherin N-terminal domain [15].

Because MPII and FRA are counter-transcribed in the bacterial genome, our novel data suggest that these two proteinases perform distinctly in the course of B. *fragilis* infection and

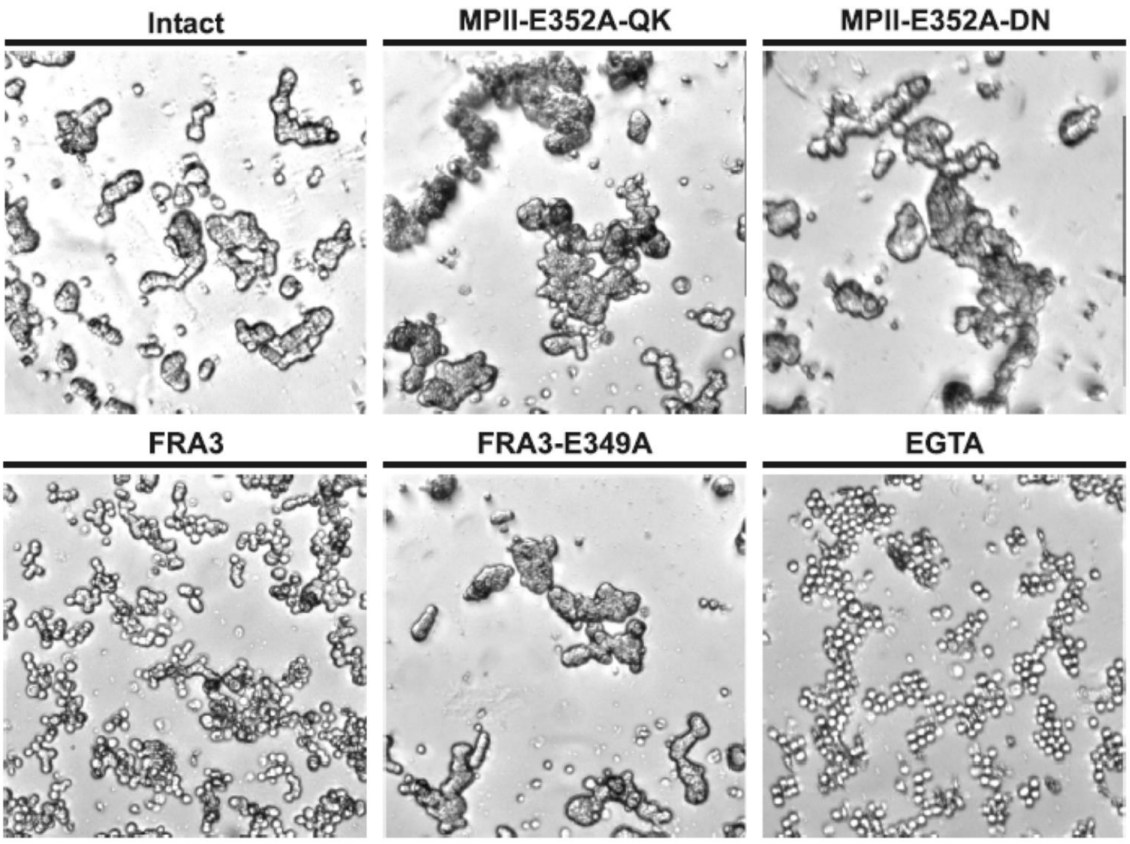

Fig. 4: The binding of MPII to E-cadherin does not interfere with cell aggregation. Bright-field microscope images of intact HT29 cells and HT29 cells co-incubated for 24 h with MPII-E352A-QK, MPII-E352A-DN, and inert FRA3-E349A (10 μg/ml each) and active FRA3 (500 ng/ml) and EGTA (3 mM; control).

that they exhibit the specialized functions. The preliminary data of our work in progress indicated that MPII binds not only the host cell E-cadherin but also the bacterium itself and that MPII may perform as a bridge between the pathogen and the host. As a result, it is possible that the putative cleavage targets of MPII could be found not only on the host cell surface but also in B. *fragilis*.

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- Oligos were synthesized by IDT Inc. (Coralville, IA, USA)

- Resulting DNA constructs were sequenced by Eton Bioscience Inc. (San Diego, CA, USA)

References

[1] Wexler HM (2007) Bacteroides: the good, the bad, and the nitty-gritty. *Clin. Microbiol. Rev.*, 20, 593-621.

[2] Dejea C, Wick E, Sears CL (2013) Bacterial oncogenesis in the colon. *Future Microbiol.*, 8, 445-460.

[3] Rabizadeh S, Rhee KJ, Wu S, Huso D, Gan CM, et al. (2007) Enterotoxigenic bacteroides fragilis: a potential instigator of colitis. *Inflamm. Bowel Dis.*, 13, 1475-1483.

[4] Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, et al. (2009) A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat. Med.*, 15, 1016-1022.

[5] Franco AA, Cheng RK, Chung GT, Wu S, Oh HB, et al. (1999) Molecular evolution of the pathogenicity island of enterotoxigenic Bacteroides fragilis strains. *J. Bacteriol.*, 181, 6623-6633.

[6] Moncrief JS, Obiso R, Jr., Barroso LA, Kling JJ, Wright RL, et al. (1995) The enterotoxin of Bacteroides fragilis is a metalloprotease. *Infect. Immun.*, 63, 175-181.

[7] Obiso RJ, Jr., Lyerly DM, Van Tassell RL, Wilkins TD (1995) Proteolytic activity of the Bacteroides fragilis enterotoxin causes fluid secretion and intestinal damage in vivo. *Infect. Immun.*, 63, 3820-3826.

[8] Sears CL (2009) Enterotoxigenic Bacteroides fragilis: a rogue among symbiotes. *Clin. Microbio. Rev.*, 22, 349-369.

[9] Goulas T, Arolas JL, Gomis-Ruth FX (2011) Structure, function and latency regulation of a bacterial enterotoxin potentially derived from a mammalian adamalysin/ADAM xenolog. *Proc. Natl. Acad. Sci. USA*, 108, 1856-1861.

[10] Shiryaev SA, Aleshin AE, Muranaka N, Kukreja M, Routenberg DA, et al. (2014) Structural and functional diversity of metalloproteinases encoded by the Bacteroides fragilis pathogenicity island. *FEBS J.*, 281, 2487-2502.

[11] Gomis-Ruth FX (2003) Structural aspects of the metzincin clan of metalloendopeptidases. *Mol. Biotechnol.*, 24, 157-202.

[12] Sanchez E, Laparra JM, Sanz Y (2012) Discerning the role of Bacteroides fragilis in celiac disease pathogenesis. *Appl. Environ. Microbiol.*, 78, 6507-6515.

[13] Wu S, Lim KC, Huang J, Saidi RF, Sears CL (1998) Bacteroides fragilis enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc. Natl. Acad. Sci. USA*, 95, 14979-14984.

[14] Wu S, Rhee KJ, Zhang M, Franco A, Sears CL (2007) Bacteroides fragilis toxin stimulates intestinal epithelial cell shedding and gamma-secretase-dependent E-cadherin cleavage. *J. Cell Sci.*, 120, 1944-1952.

[15] Hartsock A, Nelson WJ (2008) Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochim. Biophys. Acta*, 1778, 660-669.

[16] Shiryaev SA, Remacle AG, Chernov AV, Golubkov VS, Motamedchaboki K, et al. (2013) Substrate Cleavage Profiling Suggests a Distinct Function of Bacteroides fragilis Metalloproteinases (Fragilysin and Metalloproteinase II) at the Microbiome-Inflammation-Cancer Interface. *J. Biol. Chem.*, 288, 34956-34967.

[17] Weikel CS, Grieco FD, Reuben J, Myers LL, Sack RB (1992) Human colonic epithelial cells, HT29/C1, treated with crude Bacteroides fragilis enterotoxin dramatically alter their morphology. *Infect. Immun.*, 60, 321-327.

[18] Sears CL, Buckwold SL, Shin JW, Franco AA (2006) The C-terminal region of Bacteroides fragilis toxin is essential to its biological activity. *Infect. Immun.*, 74, 5595-5601.

[19] Holton J (2008) Enterotoxigenic Bacteroides fragilis. *Curr. Infect. Dis. Rep.*, 10, 99-104.

[20] Buckwold SL, Shoemaker NB, Sears CL, Franco AA (2007) Identification and characterization of conjugative transposons CTn86 and CTn9343 in Bacteroides fragilis strains. *Appl. Environ. Microbiol.*, 73, 53-63.

[21] Franco AA (2004) The Bacteroides fragilis pathogenicity island is contained in a putative novel conjugative transposon. *J. Bacteriol.*, 186, 6077-6092.

[22] Franco AA, Cheng RK, Goodman A, Sears CL (2002) Modulation of bft expression by the Bacteroides fragilis pathogenicity island and its flanking region. *Mol. Microbiol.*, 45, 1067-1077.

[23] Chung GT, Franco AA, Wu S, Rhie GE, Cheng R, et al. (1999) Identification of a third metalloprotease toxin gene in extraintestinal isolates of Bacteroides fragilis. *Infect. Immun.*, 67, 4945-4949.

[24] Franco AA, Buckwold SL, Shin JW, Ascon M, Sears CL (2005) Mutation of the zinc-binding metalloprotease motif affects Bacteroides fragilis toxin activity but does not affect propeptide processing. *Infect. Immun.*, 73, 5273-5277.

[25] Franco AA, Mundy LM, Trucksis M, Wu S, Kaper JB, et al. (1997) Cloning and characterization of the Bacteroides fragilis metalloprotease toxin gene. *Infect. Immun.*, 65, 1007-1013.

[26] Kharlampieva DD, Manuvera VA, Podgorny OV, Kovalchuk SI, Pobeguts OV, et al. (2013) Purification and characterisation of recombinant Bacteroides fragilis toxin-2. *Biochimie*, 95, 2123-2131.

[27] Kling JJ, Wright RL, Moncrief JS, Wilkins TD (1997) Cloning and characterization of the gene for the metalloprotease enterotoxin of Bacteroides fragilis. *FEMS Microbiol. Lett.*, 146, 279-284.

[28] Nakano V, Gomes DA, Arantes RM, Nicoli JR, Avila-Campos MJ (2006) Evaluation of the pathogenicity of the Bacteroides fragilis toxin gene subtypes in gnotobiotic mice. *Curr. Microbiol.*, 53, 113-117.

[29] Riegler M, Lotz M, Sears C, Pothoulakis C, Castagliuolo I, et al. (1999) Bacteroides fragilis toxin 2 damages human colonic mucosa in vitro. *Gut*, 44, 504-510.

[30] Van Tassell RL, Lyerly DM, Wilkins TD (1992) Purification and characterization of an enterotoxin from Bacteroides fragilis. *Infect. Immun.*, 60, 1343-1350.

[31] Wu S, Shin J, Zhang G, Cohen M, Franco A, et al. (2006) The Bacteroides fragilis toxin binds to a specific intestinal epithelial cell receptor. *Infect. Immun.*, 74, 5382-5390.

[32] Remacle AG, Shiryaev SA, Strongin AY. (2014) Distinct interactions with cellular E-cadherin of the two virulent metalloproteinases encoded by a Bacteroides fragilis pathogenicity island, submitted for publication. *PLoS One*, 9, e113896.