Microbial Pathogenesis 83-84 (2015) 47–56

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Recombinant fragilysin isoforms cause E-cadherin cleavage of intact cells and do not cleave isolated E-cadherin

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ARTICLE INFO

Article history:
Received 30 January 2015
Received in revised form
28 April 2015
Accepted 15 May 2015
Available online 18 May 2015

Keywords:
Bacteroides fragilis
Metalloprotease
BFT
Fragilysin
E-cadherin

ABSTRACT

The fragilysin (BFT) is a protein secreted by enterotoxigenic *Bacteroides fragilis* strains. BFT contains zinc-binding motif which was found in the metzincins family of metalloproteinases. In this study, we generated three known recombinant isoforms of BFT using *Escherichia coli*, tested their activity and examined whether E-cadherin is a substrate for BFTs. BFT treatment of HT-29 cells induced endogenous E-cadherin cleavage, and this BFT activity requires the native structure of zinc-binding motif. At the same time recombinant BFTs did not cleave recombinant E-cadherin or E-cadherin in isolated cell fractions. It indicates that E-cadherin may be not direct substrate for BFT. We also detected and identified proteins released into the cultural medium after HT-29 cells treatment with BFT. The role of these proteins in pathogenesis and cell response to BFT remains to be determined.

1. Introduction

Colonization of human body by microorganisms begins immediately after birth, and many of these microorganisms become really essential to the host. Bacteria play a major role in processes of nutrition, digestion and immunity. The majority of microorganisms colonizing a colon are anaerobes, and about of 25% of them are species of *Bacteroides* genus [1]. *Bacteroides* are gram-negative bile-resistant non-spore-forming rods. *Bacteroides fragilis* normally presents in intestinal flora and participates in carbohydrate fermentation and biotransformation of bile acids [2]. The population of *B. fragilis* comprise 1–10% of the total intestinal population of bacteria from *Bacteroides* genus [2]. However, when *B. fragilis* escapes the gastrointestinal tract, it may cause significant pathology. The capsule of *B. fragilis* initiates an abscess formation [3]. Abscesses without treatment may expand and rupture, resulting in bacteremia. *B. fragilis* is the most common organism accompanying bloodstream infections and abdominal abscesses among *Bacteroides* species. *B. fragilis* attracted interest of researchers because it was revealed the association of the bacterium with acute diarrheal disease in newborn lambs, which was accompanied by inappetance, depression and a high mortality rate [4]. In their study Myers and colleagues [4] found that some isolates of *B. fragilis* stimulated fluid accumulation into ligated intestinal loop of lambs and calves. *B. fragilis* strains inducing fluid accumulation into ligated intestinal loop were termed enterotoxigenic *B. fragilis* (ETBF) in contrast to non-toxigenic strains (NTBF) which are lacked this ability. In 1987, enterotoxigenic *B. fragilis* strains were isolated from the human diarrhoea stool specimens [5]. Till the study of Myers and colleagues [4] *B. fragilis* had not been reported to cause fluid accumulation in the intestine or to cause diarrhea in any species of animal. Because the profuse watery diarrhea indicate enterotoxin type of mediation, the authors decided to test whether ETBF secreted protein with ability to cause fluid accumulation in ligated intestinal loop. They provided the first evidence that ETBF

Abbreviations: BFT, *Bacteroides fragilis* toxin; mBFT, mature BFT; prBFT, proform of BFT consisting of prodomain and catalytic domain; ETBF, enterotoxigenic *Bacteroides fragilis*; NTBF, non-toxigenic *Bacteroides fragilis*.

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http://dx.doi.org/10.1016/j.micpath.2015.05.003
0882-4010/

secreted a heat-labile protein stimulating intestinal secretion [6]. Further, this protein (termed fragilysin, or BFT – *B. fragilis* toxin) was isolated. It was demonstrated that isolated BFT stimulates intestinal secretion and causes intestinal damage, neutrophilic inflammation and in some cases necrosis and hemorrhage [7].

BFT is a secreted protein encoded by gene into 6 kb pathogenicity island [8]. BFT is synthesised as a preproprotein containing a signal peptide and two domains. During maturation the signal peptide and N-terminal domain (prodomain) are cleaved. Remaining C-terminal domain (catalytic domain) is the mature form of the toxin [9]. Catalytic domain contains the HEXXHXXGXXH motif, which is a zinc-binding motif found in the metzincins family of metalloproteinases [10,11]. Prodomain inhibits activity of catalytic domain [12]. Three isoforms of the BFT with several differences in their primary structures were identified [9,13–15]. Differences in the sequences of BFT-1, BFT-2 and BFT-3 were observed in 2–5 positions in N-terminal domain and in up to 25 positions in the C-terminal domain.

It was demonstrated, that all isoforms of BFT destroy the zonula adherens tight junctions in intestinal epitelium by inducing E-cadherin cleavage, resulting in actin cytoskeleton rearrangements [16,17]. Loss of tight junction leads to entry of fluid into intestinal lumen. This may explain diarrhoea caused by ETBF [17]. On the other side BFT treatment of cells may induce transcription of new genes. It was demonstrated, that BFT treatment of cells leads to synthesis of proinflammatory cytokines (IL-8, MCP-1, CXCL1) [18]. Also BFT-induced E-cadherin cleavage promotes colonic permeability and access of innate mucosal immune cells to luminal bacterial antigens. This possibly promotes mucosal inflammatory and secretory responces [19,20]. Moreover, cleaved E-cadherin fragments possess the oncogenic role [21]. Moreover, in other studies it has been found that cleavage of E-cadherin, induced by BFT-2, releases β-catenin. Then β-catenin nuclear translocation leads to expression of proto-oncogene *C-myc* and cell proliferation [22]. Thus data indicating possible role of BFT in mucosal inflammation and colorectal cancer formation are accumulated. Because BFT is a secreted protein with zinc-binding motif which is specific for metzincins family of metalloproteinases and because BFT treatment of HT-29 cells induced E-cadherin cleavage, the goal of our study was to investigate whether BFT cleaves E-cadherin directly. Direct interaction between E-cadherin and BFT was not demonstrated before.

To this end we generated wild-type recombinant isoforms of BFTs and BFTs with mutated HEXXHXXGXXH motif (glutamic acid residue mutated to an alanine residue and with zinc ion-chelating histidine residues substituted to tyrosine residues). We tested the recombinant proteins using HT-29 cells to reveal whether E-cadherin cleavage occurs. We also examined recombinant BFTs using azocoll, azocasein and gelatin. Proteolysis of this substrates by BFTs 1 and 3 was described previously [11,12]. Moreover, we tested activity of recombinant BFTs using recombinant thioredoxins containing linker which are potentially cleaved by BFT according to Shiryaev S.A. [23]. We produced recombinant E-cadherin in *Escherichia coli* and Expi293F cells and isolated an enriched fraction of HT-29 cell membrane proteins and suspension of the enriched membrane fraction. We examined whether E-cadherin isolated from these sources is cleaved by recombinant BFTs. Finally, to find new potential substrates for BFT we identified proteins released in culture medium after BFT treatment of HT-29 cells.

2. Materials and methods

2.1. Isolation of DNA fragments encoding prBFTs 1 and 3

To obtain DNA encoding proform of BFT 1 and 3, consisting of prodomain and catalytic domain (prBFT) we performed screening of DNA from faeces samples. Biological material was obtained according to guidelines of the ethics committee of Scientific Research Institute of Physical-Chemical Medicine. We performed DNA isolation from faeces as it was described previously [24].

We used nested PCR to identify sequences encoding BFTs 1 and 3 in faecal DNA. For the first round of nested PCR, we used primers u for 601 – u rev and for the second round of nested PCR, we used primers u for 632 – u rev 1000 (Table S1). The resulting PCR product was isolated by preparative electrophoresis. Nucleotide sequences of the fragments were identified by sequencing On AbiPrism 3730xl platform (Applied Biosystems, USA) using the BigDye Terminator Cycle Sequencing Kit (v. 3.1) and AbiPrism 3730xl (Applied Biosystems, USA). DNA samples containing sequences encoding BFT isoforms 1 and 3 were used to obtain full-length fragments encoding prBFTs. The first round of nested PCR was performed with primers nestF and nestR and for the second round we used primers pBft-Bgl – C-Bft-Sal1iso for prBFT 1 and pBft-Bgl – C-Bft-Sal for prBFT 3 (Table S1). Detailed methods are described in the Supplementary (see Sections 1–4).

2.2. 6xHisTag prBFTs-1, 2, 3 plasmid construction

We generated the recombinant plasmids encoding full-length prBFT 1, 2 and 3 (without signal peptide) fused with the signal peptide of fd phage GIII protein and a C-terminal 6xHisTag (pBAD/GIII-prBft1, pBAD/GIII-prBft, pBAD/GIII-prBft3). Generation of the recombinant plasmid pBAD/GIII-prBft encoding full-length prBFT was described previously [25]. Construction of the plasmids encoding prBFT 1 and prBFT 3 was performed as described previously for pBAD/GIII-prBft [25]. Isolated DNA fragments encoding prBFTs 1 and 3 (see 2.6. Isolation of DNA fragments encoding prBFTs 1 and 3) were used. The resulting plasmids were named pBAD/GIII-prBft1 and pBAD/GIII-prBft3, respectively.

We also generated plasmid pETmin/prBFT-His coding for full-length prBFT-2 (without signal peptide) fused with C-terminal 6xHis Tag. We performed PCR using the plasmid pBAD/GIII-prbft as a template and BAD F and BAD R as primers. Amplicons were cloned into pETmin vector. Plasmid pETmin was derived from the commercial plasmid pET-22b(+) (Novagen, USA). Detailed methods and the plasmid maps (Figs. S1, S2, S3) are represented in the Supplementary (Section 5).

2.3. Untagged prBFT-1, 2, 3 plasmid construction

To investigate the effect of the polyhistidine sequence on protein activity and compare isoforms' digestion activity, we generated recombinant wild-type BFTs (three known isoforms) without a 6xHis Tag. We generated plasmids pBAD/GIII-prBft1-min, pBAD/GIII-prBft2-min, pBAD/GIII-prBft3-min encoding untagged prBFTs-1, 2, 3 fused to the fd phage GIII protein signal peptide. To this end we performed PCR using pBAD/GIII-prBft and pBAD/GIII-prBft3 as templates and pBft-Bgl and C BFT min as primers. In the case when pBAD/GIII-prBft1 was used as template for PCR, we changed C BFT min primer to C BFT m1 primer. These primers allowed to introduce stop codon before the sequence encoding 6xHis Tag. The remaining preparation steps were as listed in 2.2.

2.4. Site-directed mutagenesis

To obtain mutant BFTs we used primers listed in Table S2. As a result of site-directed mutagenesis we obtained the plasmids encoding the following mutant prBFTs: (i) the catalytic glutamic acid residue mutated to alanine (E349A): pBAD/GIII-prBft1-E349A, pBAD/GIII-prBft2-E349A, pBAD/GIII-prBft3-E349A; (ii) the zinc-chelating histidine residues mutated to tyrosine residues: pBAD/GIII-prBft2-HY (H348Y, H352Y, H358Y).