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Pathogenesis and toxins

Determination of the *Clostridium perfringens*-binding site on fibronectin

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ABSTRACT

The extracellular matrix protein fibronectin (Fn) is known to bind to the surface of *Clostridium perfringens* cells. Fn is a disulfide-linked homodimer protein, with each Fn polypeptide consisting of three types of repeating modules: 12 type I, 2 type II, and 15-17 type III modules. To determine the epitope on Fn recognized by *C. perfringens* cells, anti-Fn monoclonal antibodies (mAbs) and various Fn fragments ($III_{2\text{-}10}$, $rIII_{2\text{-}4}$, $rIII_{5\text{-}7}$, $rIII_8$, $rIII_9$, $rIII_{10}$) were employed. Although two *C. perfringens*-derived Fn-binding proteins, FbpA and FbpB, have been reported, they appear not to be the bacterium's surface Fn receptor. Moreover, both FbpA and FbpB were found to bind to *C. perfringens* cells. To avoid confusion, a mutant *C. perfringens* lacking both the *fbpA* and *fbpB* genes (MW5) was prepared using an in-frame deletion system. MW5 cells bound Fn on their surface, suggesting the presence of a putative Fn receptor(s) on *C. perfringens* cells. Of several anti-Fn mAbs, both HB39 and MO inhibited the binding of Fn to MW5 cells. HB39 reacted strongly with $III_{2\text{-}10}$ and $rIII_9$, and weakly with $rIII_{2\text{-}4}$, $rIII_{10}$ and $rIII_{5\text{-}7}$ in Western blotting analysis. Binding of HB39 to Fn was inhibited in the presence of either $rIII_9$ or $rIII_{10}$, but not in the presence of $rIII_{2\text{-}4}$, $rIII_{5\text{-}7}$, or $rIII_8$. Binding of Fn to MW5 cells was strongly inhibited by both $III_{2\text{-}10}$ and $rIII_9$, marginally inhibited by $rIII_{2\text{-}4}$, but not affected by $rIII_{5\text{-}7}$, $rIII_8$, or $rIII_{10}$. Significant binding of MW5 cells to immobilized $rIII_9$ and $rIII_{10}$ as well as immobilized $III_{2\text{-}10}$ was observed. The region of Fn recognized by *C. perfringens* was thus mapped to the region encompassed by III_9 and III_{10}.

1. Introduction

Fibronectin (Fn), existing either as an insoluble extracellular matrix glycoprotein or as a soluble plasma protein, is abundantly found in tissues and blood. Fn plays a role in many cellular processes, including wound healing, tissue structure formation, and cell migration [1]. Fn is a disulfide-linked homodimer glycoprotein, with each Fn polypeptide consisting of three types of repeating modules: 12 type I, 2 type II, and 15-17 type III modules (Fig. 1). The N-terminal domain is composed of 5 type I modules ($I_{1\text{-}5}$), where most bacterial Fn-binding proteins (Fbps) are known to bind [2], and an adjacent gelatin-binding domain containing 4 type I modules and 2 type II modules ($I_6II_{1\text{-}2}I_{7\text{-}9}$). The central part of the Fn polypeptide is composed of 15–17 type III modules, containing the cell-binding region (III_{10}). In contrast to both type I and type II modules, type III modules lack intramodular disulfide bonds.

Many pathogenic bacteria encode Fbps [3], taking advantage of Fn to promote colonization and host cell contact. *Clostridium perfringens* is an obligate anaerobe that causes a serious wound-associated infection as well as food poisoning. Binding of *C. perfringens* to Fn was recently reported [4]. Two Fn-binding proteins of *C. perfringens*, FbpA and FbpB, were identified based upon genetic similarity to the Fn-binding proteins of *Listeria monocytogenes* and *Clostridium difficile*, respectively [5].

The recombinant forms of FbpA and FbpB (rFbpA and rFbpB) bind to Fn, recognizing the III_1-C region of Fn, which is known to be cryptic [6]. Consistent with this observation, only 1% of serum Fn was found to bind rFbps [6]. Both rFbp-binding Fn (1% of serum Fn) and rFbp-nonbinding Fn (99% of serum Fn) were found to bind to *C. perfringens* cells (data not shown). However, Fn-binding to *C. perfringens* is competitively inhibited in the presence of soluble

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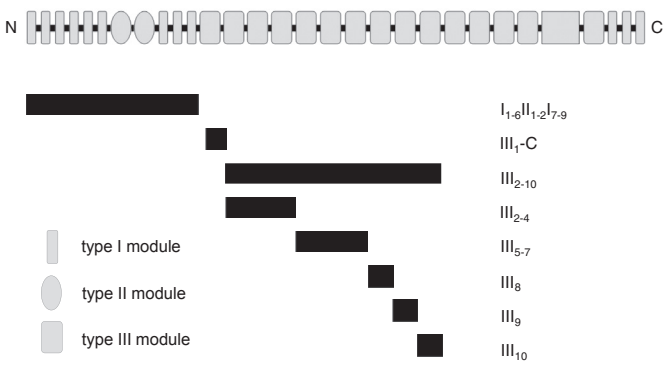

Fig. 1. Schematic representation of the modular structure of Fn.

rFbps [5]. These findings strongly suggest that competitive inhibition of the binding of Fn to *C. perfringens* cells occurs at the surface Fn receptor level. In fact, rFbps bind to *C. perfringens* cells [7], suggesting that they bind to the surface Fn receptor of *C. perfringens*.

An important question regarding the binding of Fn to *C. perfringens* cells is the site on the Fn molecule that recognizes the bacterial Fn receptor. Because leaked intracellular FbpA and FbpB may bind to the putative surface Fn receptor of *C. perfringens*, the experimental results obtained using wild-type *C. perfringens* were confusing. To avoid such ambiguity in this study, we prepared a mutant *C. perfringens* strain (MW5) that lacks both FbpA and FbpB. We then sought to determine the Fn region that binds the putative surface Fn receptor of *C. perfringens*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli NovaBlue and BL21-CodonPlus® (DE3) RIL (Stratagene, La Jolla, CA) were used as recipient strains for transformation and overexpression of recombinant proteins. Bacteria were grown in Luria–Bertani (LB) broth (Invitrogen, Carlsbad, CA) or on LB agar plates. *C. perfringens* Type A strain 13, isolated from human gas gangrene, was mainly used in this work. *C. perfringens* HN13 (strain13 *ΔgalK ΔgalT*) [8] was used for knockout of both the *fbpA* and *fbpB* genes. All of the *C. perfringens* strains were anaerobically grown in Gifu anaerobic medium (GAM) (Nissui Co., Tokyo, Japan) using an AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan). The following antibiotics were used at the indicated concentrations: ampicillin (50 μg/mL) and chloramphenicol (Cm, 10, 20 or 34 μg/mL).

2.2. DNA manipulations and RT-PCR

Chromosomal DNA from the *C. perfringens* strains was purified as previously described [8]. Isolation of plasmid DNA from *E. coli* was performed using the Wizard DNA purification kit according to the manufacturer's protocol (Promega, Madison, WI). Transformation of *E. coli*, agarose gel electrophoresis, and DNA recombination were carried out as previously described [9]. The nucleotide sequences of cloned genes were determined using a DNA sequencer (model 310, PE Applied Biosystems, Foster City, CA). Polymerase chain reaction (PCR) analyses were performed using Takara Ex *Taq*® or PrimeSTAR® HS DNA polymerase (Takara, Kyoto, Japan). RT-PCR analyses of *fbpA*, *fbpB* and *plc* gene as a positive control were basically carried out as described previously [5]. Total RNA was extracted from *C. perfringens* cells at an OD_{600} of 0.8 (exponential phase). One or 2 μg of total RNA was used in each RT-PCR. The primers used for the RT-PCRs are listed in Table 1. The sequences of these primers corresponded to the beginning and ending sites of the open reading frames of the genes.

2.3. In-frame deletion of fbpA and fbpB

Both *fbpA* and *fbpB* were deleted using an in-frame (IF) deletion system, basically as reported by Nariya et al. [8]. First, the 1-kb upstream and 1-kb downstream regions of *fbpA* were amplified by PCR using chromosomal DNA of *C. perfringens* strain 13 as a template along with appropriate primers, and the resulting amplicons were then cloned in tandem into the *Bam*HI site and *Pst*I site of pGALK, a suicide vector for IF deletion [8] (Fig. 2A). The resultant plasmid, pYS3, contained the 1-kb upstream and 1-kb downstream sequence of the *fbpA* gene. *C. perfringens* HN13 was then transformed with pYS3 by electroporation [10]. As pGALK contains a Cm-resistance gene and there is no *C. perfringens* replication origin in this plasmid, the transformants were generated through single-crossover recombination at the upstream or downstream region of the *fbpA* gene after overnight growth on 1/2GAM-Cm (10 μg/mL) at 37 °C. Then, the transformants were grown on 1/2TYGA3 (1.5% [w/v] tryptone, 1% [w/v] yeast extract, 0.1% [w/v] thioglycolate, 3% [w/v] galactose) agar plates overnight at 37 °C. The colonies that arose on the plate were considered to have excised the chromosomal pGALK region containing the *galK-Ca* (*Clostridium acetobutylicum*) [8] and Cm^r genes through a second single-crossover recombination event because the GalK product was toxic to the Δ*galT* Δ*galK* mutant (HN13). Next, to confirm that the pGALK region was excised from the chromosome, colonies sensitive to Cm (20 μg/mL) were selected. The resultant strains were revertants or Δ*fbpA* mutants. IF deletion mutants of *fbpA* (HN13 Δ*fbpA*) were determined by colony PCR using appropriate primers. One of the IF deletion mutants was designated MW4.

To construct an *fbpA* and *fbpB* double-knockout mutant, *fbpB* was deleted in MW4 using the IF deletion system, basically as described above. The 1-kb upstream and 1-kb downstream regions of *fbpB* were amplified by PCR using chromosomal DNA of strain 13 as a template along with appropriate primers, and the resulting amplicons were cloned in tandem into the *Bam*HI site and *Pst*I site of pGALK. The resultant plasmid, pMM3, contained the 1-kb upstream and 1-kb downstream sequence of *fbpB* gene. *C. perfringens* MW4 was then transformed with pMM3 by electroporation. Transformants resistant to Cm (10 μg/mL) were grown on 1/2TYGA3 agar plates overnight at 37 °C. The colonies that arose on the plate were considered to have excised the chromosomal pGALK region containing the *galK-Ca* and Cm^r gene. Then, colonies sensitive to Cm (20 μg/mL) were selected. As these strains were revertants (Δ*fbpA*) or alternatively, Δ*fbpA* Δ*fbpB* double mutants, IF deletion mutants of both *fbpA* and *fbpB* were determined by colony PCR using appropriate primers. The IF deletion mutant was designated MW5, and it was confirmed that this mutant possessed deletions of both *fbpA* and *fbpB* using Southern blot hybridization (data not shown).

2.4. Preparation of Fn and Fn fragments

Fn was purified from pooled human serum using a gelatin-Sepharose column, as described previously [6]. The modular structure of the Fn molecule is schematically represented in Fig. 1. The proteolytic N-terminal 70-kDa Fn fragment ($I_{1-6}II_{1-2}I_{7-9}$) was purchased from Sigma (St. Louis, MO). The 110-kDa Fn fragment (III_{2-10}) was obtained by digestion of Fn with thermolysin (Sigma), followed by gel-filtration chromatography on a HiLoad 16/60