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Research paper
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Expression of glyceraldehyde-3-phosphate dehydrogenase on the surface of *Clostridium perfringens* cells

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ABSTRACT

During research to identify fibronectin (Fn)-binding proteins (Fbps) on the surface of *Clostridium perfringens* cells, we identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a candidate Fbp. GAPDH is a glycolytic enzyme found in a wide range of prokaryotes and eukaryotes. The Fn-binding activity of recombinant *C. perfringens* GAPDH (rGAPDH) was investigated using both ligand blotting analysis and enzyme-linked immunosorbent assay (ELISA). rGAPDH strongly bound plasminogen but not laminin or gelatin. Although GAPDH has no signal sequence, it is expressed on the cell surface of many microorganisms. The presence of GAPDH on the surface of *C. perfringens* cells was analyzed using ELISA and flow cytometry analyses; purified rGAPDH bound to the surface of *C. perfringens* cells. As autolysin is reportedly involved in the binding of GAPDH to the cell surface, we evaluated the interaction between rGAPDH and the *C. perfringens* autolysin Acp by both ELISA and ligand blotting assay. These assays revealed that rGAPDH binds to the catalytic domain of Acp but not the cell wall binding domains. These results suggest that autolysin mediates expression of GAPDH on the surface of *C. perfringens* cells and indicate a possible moonlighting function for GAPDH in binding both Fn and plasminogen.

1. Introduction

Clostridium perfringens is a Gram-positive, spore-forming anaerobic bacterium that causes food poisoning and gas gangrene. Infections with *C. perfringens* are characterized by associated tissue injury and rapid growth of the bacterium in the infected region [1]. The virulence of *C. perfringens* is associated with its ability to adhere to injured tissue and produce various toxins. Both the adherence of bacteria to tissues and subsequent colonization play important roles in the establishment and persistence of *C. perfringens* infections. Binding of fibronectin (Fn) to collagen mediates adhesion of *C. perfringens* cells to connective tissues [2]. Thus, Fn receptors on the surface of *C. perfringens* cells play a pivotal role in bacterial colonization and virulence.

In previous research aimed at identifying *C. perfringens* peptidoglycan-associated Fn-binding proteins (Fbps), we identified two Fbps, FbpC and FbpD [3]. Ligand-blotting assay results indicated a faint band as a potential third Fbp. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of the protein indicated that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) could be the third peptidoglycan-associated Fbp in *C. perfringens* (Supplementary Figure S1).

GAPDH is a representative 'moonlighting' protein, which refers to a single protein that has multiple functions. GAPDH is expressed on the cell surface of many microorganisms, including *Candida albicans*, *Streptococcus pyogenes*, *Trichomonas vaginalis*, *Bacillus anthracis*, *Lactobacillus casei*, *L. plantarum*, *Penicillium marneffei*, *Str. pneumoniae*, and *Staphylococcus aureus* [4–12]. These GAPDHs bind to various host extracellular matrix proteins,

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including Fn, laminin, fibrinogen, plasminogen, and collagen. Interestingly, the cell surface–expressed GAPDH of *S. aureus* was recently reported as binding both plasminogen and fibrinogen in addition to the major autolysin (Atl), an *N*-acetylmuramyl-L-alanine amidase, and a glucosaminidase [12]. *S. aureus* Atl is a cell wall–associated enzyme located at the septum of dividing cells that plays a role in mediating the separation of daughter cells [13]. This suggests that expression of GAPDH on the surface of *S. aureus* cells involves interaction with Atl. Thus, whether recombinant GAPDH (rGAPDH) of *C. perfringens* also binds autolysin is of interest. The *C. perfringens* autolysin Acp, composed of a C-terminal catalytic domain (CD) exhibiting *N*-acetylglucosaminidase activity and 10 SH modules of cell wall binding domain (CWB) and a signal peptide in the N-terminus, is involved in cell wall reconstitution during cell separation [14]. The structure and reaction mechanism of the CD of Acp have been analyzed in detail [15]. Here, we report the Fn-binding activity, cell surface expression, binding to the autolysin Acp of *C. perfringens* GAPDH.

2. Materials and methods

2.1. Bacterial strains and growth conditions

C. perfringens type A strain 13 and *C. perfringens* HN13 (strain 13 Δ*galK* Δ*galT*) [16] were grown anaerobically in Gifu anaerobic medium (GAM) (Nissui Co., Tokyo, Japan) using an Anaero Pack system (Mitsubishi Gas Chemical, Tokyo, Japan). *Escherichia coli* DH5α, *E. coli* NovaBlue, and *E. coli* DH10B were used for construction of plasmids.

2.2. Preparation of recombinant proteins and Fn

Strain BL21-CodonPlus (DE3) RIL (Stratagene, La Jolla, CA) harboring the plasmids pNAK1 [3], pNAK2 [3], and pNAY1 was used for expression of N-terminal × 6His-tagged rFbpC, rFbpD, and rGAPDH, respectively. For the details regarding construction of pNAY1, refer to the Supplementary Information (Supplementary Table S1). All strains were cultured at 37 °C in LB broth containing appropriate antibiotics. When the optical density at 600 nm (OD_{600}) was approximately 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After incubation for 3 h at 37 °C, the cells were harvested and lysed using a French press (10,000 psi). Proteins were purified on separate Ni^{2+}-Sepharose columns (GE Healthcare, Fairfields, CT). Final purified proteins were dialyzed against 10 mM veronal-buffered saline (pH 7.4) (VBS).

Strain BL21-CodonPlus RIL (Stratagene) harboring the plasmids pColdAcpCD [15], pColdAcpHCWB3, and pColdAcpCWB1-6H was used to express the N-terminal × 6His-tagged AcpCD, C-terminal × 6His-tagged 8 CWB domains with CD (AcpCWB3-10 + CD), and C-terminal × 6His-tagged 6 CWB domains (AcpCWB1-6), respectively. For details regarding the newly constructed plasmids, refer to the Supplementary information (Fig. S2). Protein expression and purification were performed according to the method described previously [15]. Finally, purified proteins were dialyzed against 20 mM Tris-HCl (pH 7.5) buffer containing 100 mM NaCl. Fn was purified from pooled human serum using a gelatin-Sepharose column, as described previously [17].

2.3. Preparation of anti-rGAPDH antibody (anti-rGAPDH Ab) and anti–C. perfringens type A strain 13 antibody (anti-st13 Ab)

C. perfringens was cultured in 20 ml of medium for approximately 2 h until reaching an OD_{600} of 0.5, at which time the cells were harvested. After several washes with VBS, the cells were lysed using a French press as described in Section 2.2 above. The lysate precipitate was then suspended in 2 ml of saline and used as the immunogen for the *C. perfringens* strain 13 cell wall fraction. Next, 2 ml of rGAPDH solution (0.5 mg/ml) or 2 ml of a suspension of *C. perfringens* strain 13 cell wall fraction was emulsified with an equal volume of complete Freund's adjuvant and subcutaneously injected into separate rabbits. After 1 month, each rabbit was boosted with a one-tenth dose of the same immunogen. One week after the secondary immunization, the rabbits were bled, and the IgG fraction was prepared from each antiserum sample using a HiTrap™ Protein A HP column (GE Healthcare). The IgG fraction containing anti-rGAPDH Ab was further purified using an rGAPDH-conjugated Sepharose column to obtain affinity-purified anti-rGAPDH Ab.

2.4. SDS-PAGE and ligand blotting analysis

Proteins were separated on a 10% SDS-PAGE gel under non-reducing conditions. The electrophoresed proteins were then either silver stained or transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked overnight in 0.4% (v/v) Tween 20 in 20 mM Tris-HCl (pH 7.4)–buffered saline, incubated in 5 ml of biotinylated Fn (30 μg/ml) or 10 ml of biotinylated rGAPDH (60 μg/ml) for 1 h at room temperature, and then treated with horseradish peroxidase (HRP)-conjugated streptavidin (1:1000) (Vector Laboratories, Burlingame, CA) in 10 mM Tris-buffered saline (pH 7.4) (TBS) for 10 min at room temperature. After washing with PBS containing 0.02% (v/v) Tween 20 (PBST), the membrane was color developed by incubation in 1.39 mM 3,3′-diaminobenzidine (Sigma) in 50 mM Tris-HCl (pH 7.4) containing 0.03% (v/v) H_2O_2. The reaction was stopped by the addition of 0.1 M citric acid containing 0.01% (w/v) NaN_3.

2.5. ELISA

All plate-binding assays were carried out in EIA plates (Corning Inc., Corning, NY) in which the wells were individually coated with purified protein including antibodies or *C. perfringens* cells. For coating with protein, 50 μl of protein solution at a concentration of 20 μg/ml in 10 mM borate buffer (pH 8.5) was added to indicated wells and incubated for 30 min at room temperature. After blocking with BSA or N101 (NOF Corp., Tokyo, Japan), 1 μg of biotinylated rGAPDH (Figs. 2 and 7) or 0–10 μg of biotinylated Fn (Fig. 3) was

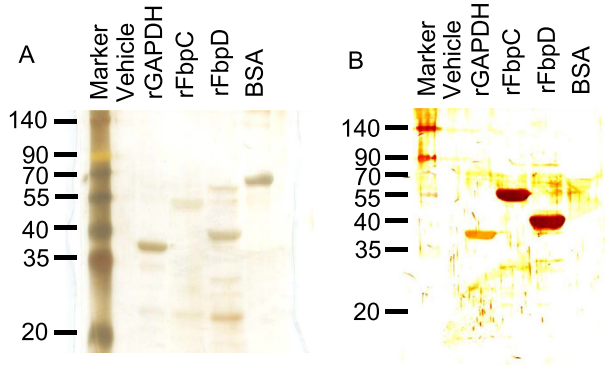

Fig. 1. SDS-PAGE and ligand blotting analyses of rGAPDH. (A) Various proteins (rGAPDH, rFbpC, rFbpD, and BSA; each 1 μg/lane) were analyzed on a 10% SDS-PAGE gel with silver staining. (B) Electrophoresed proteins were transferred onto a PVDF membrane and subjected to ligand blotting analysis using biotinylated Fn. 'Vehcle' means SDS-PAGE sample buffer.