Localization of regions of a *Bordetella pertussis* autotransporter, Vag8, interacting with C1 inhibitor

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Abstract

Bordetella pertussis is the causative agent of pertussis (whooping cough), a contagious respiratory disease that has recently seen a resurgence despite high vaccination coverage, necessitating improvement of current pertussis vaccines. An autotransporter of B. pertussis, virulence-associated gene 8 (Vag8), has been proposed as an additional component to improve pertussis vaccines. Vag8 is known to play a role in evasion of the complement system and activation of the contact system by inactivating the complement regulating factor, C1 inhibitor (C1 Inh), which inhibits serine proteases, such as plasma kallikrein (PK). However, the nature of the molecular interaction between Vag8 and C1 Inh remains to be determined. In the present study, we attempted to determine the minimum region of Vag8 that interacts with C1 Inh by examining the differently–truncated Vag8 derivatives for the ability to bind and inactivate C1 Inh. The region of Vag8 from amino–acid residues 102 to 548 was found to bind C1 Inh and cancel its inhibitory action on the protease activity of PK at the same level as a Vag8 fragment from amino–acid residues 52 to 648 covering the passenger domain, which carries its extracellular function. In contrast, the truncated Vag8 containing amino–acid residues 102 – 479 or 202 – 648 barely interacted with C1 Inh. These results indicated that the two separate regions of amino–acid residues 102 – 202 and 479 – 548 are likely required for the interaction with C1 Inh.

Importance

Pertussis is currently reemerging worldwide, and is still one of the greatest disease burdens in infants. B. pertussis produces a number of virulence factors, including toxins, adhesins, and autotransporters. One of the autotransporters, Vag8, which binds and inactivates the complement
regulator C1 Inh, is considered to contribute to the establishment of *B. pertussis* infection.

However, the nature of the interaction between Vag8 and C1 Inh remains to be explored. In this study, we narrowed down the region of Vag8 that interacts with C1 Inh and demonstrated that at least two separate regions of Vag8 are necessary for the interaction with C1 Inh. Our results provide insight into the structure–function relationship of the Vag8 molecule and information to determine its potential role in the pathogenesis of *B. pertussis*.
Bordetella pertussis causes pertussis (whooping cough), a contagious respiratory disease that has recently seen a resurgence despite high vaccination coverage (1, 2), which has prompted attempts to improve current pertussis vaccines. A number of groups have attempted to identify novel bacterial components that confer efficient immunity against B. pertussis infection, and proposed virulence-associated gene 8 (Vag8) as a possible candidate (3, 4). Vag8 is an autotransporter, which is autonomously secreted by an intramolecular system consisting of passenger and translocator domains. After passing through the inner membrane by a canonical secretion system, the passenger domain is translocated across the outer membrane by the translocator domain. The passenger domain of Vag8 is cleaved and liberated into the extracellular milieu (5). In B. pertussis infection, the liberated Vag8 binds and inactivates the complement regulating factor, C1 inhibitor (C1 Inh), which inhibits serine proteases involved in the complement system and the plasma kallikrein (PK)-kinin system (6), and plays a role in evasion of the complement system and activation of the contact system by the bacterium (5, 7, 8). However, the nature of the molecular interaction between Vag8 and C1 Inh remains unknown. Here, we attempted to localize the minimum region of Vag8 that interacts with C1 Inh by examining different truncated Vag8 derivatives for the ability to bind and inactivate C1 Inh. Our results suggest that at least two separate regions of Vag8 are necessary for interaction with C1 Inh.

Binding of truncated Vag8 derivatives to C1 Inh. We generated 9 types of truncated Vag8 derivatives together with the wild-type, Vag8WT, covering the passenger domain (Fig. 1A). The mobility of each recombinant protein on SDS-PAGE followed by immunoblotting corresponded...
to that estimated from its molecular mass (Fig. 1B). The ability of each recombinant Vag8 to
bind C1 Inh was quantified by ELISA-based binding assay. Among the C-terminal deletion
mutants, Vag852-596 and Vag852-548 bound C1 Inh to similar extents to Vag8WT, while the binding
of Vag852-479 was markedly reduced (Fig. 2A, left panel). The N-terminally deleted derivatives
showed decreased binding levels with extension of deletion (Fig. 2A, center panel).
Both-terminal deletion derivatives, Vag8102-596 and Vag8102-548, bound C1 Inh similarly to
Vag8WT, while the binding of Vag8102-479 was markedly diminished (Fig. 2A, right panel). Taken
together, these observations indicated that the region from amino–acid residues 102 to 548 is a
prerequisite for binding to C1 Inh.

Cancellation of inhibitory effects of C1 Inh by Vag8s. The interactions between C1 Inh and
the Vag8 derivatives were also examined based on the inhibitory effect of C1 Inh on the protease
activity of PK (7, 9). Vag852-596, Vag852-548, Vag8102-648, Vag8102-596, and Vag8102-548 inhibited the
C1 Inh activity to the same level as Vag8WT, while Vag852-479, Vag8202-648, and Vag8102-479, which
scarcely bound to C1 Inh, did not (Fig. 2B). Vag8152-648, which showed intermediate binding to
C1 Inh, did not inhibit the C1 Inh function. In addition, we examined whether the truncated Vag8
derivatives inhibit formation of the complex of PK and C1 Inh, which was detected by mobility
shifts on SDS-PAGE followed by immunoblotting. The truncated Vag8 derivatives carrying the
minimum region for association with C1 Inh inhibited covalent binding between PK and C1 Inh,
while the others did not (Fig. 2C).

Conclusions. We demonstrated that the region of Vag8 consisting of amino–acid residues 102 –
548 retains the ability to bind C1 Inh and to cancel its inhibitory action on PK. As neither
Vag8-102-479 nor Vag8-202-648 showed the full activity of intact Vag8, the regions of amino–acid residues 102 – 202 and 479 – 548 are likely required for the interaction with C1 Inh, suggesting that at least two separate regions of Vag8 mediate the interaction with C1 Inh. Although further studies are required, the present study provide insight into the mechanism by which Vag8 inhibits C1 Inh activity.

Methods. (i) Recombinant Vag8 derivatives. DNA fragments encoding WT and truncated derivatives of Vag8 were amplified from B. pertussis 18323 using appropriate primers (Table S1 in the supplemental material), and cloned into the XhoI-EcoRI or XhoI-HindIII sites of pCold II-HAT (3). The expression of each Vag8 derivative from Escherichia coli BL21 (DE3) or DH5α harboring each expression vector was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside. The collected bacteria were lysed in 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl (Buffer A) by sonication. After centrifugation, the pellets were dissolved in Buffer A containing 8 M urea and dialyzed against Buffer A. The resultant samples were centrifuged, and the supernatants were independently applied to a column of HIS-Select Nickel Affinity Gel (Sigma-Aldrich) equilibrated with 50 mM sodium phosphate buffer, pH 4.5, containing 300 mM NaCl (Buffer B). After nonabsorbed substances had been washed out of the column with Buffer B, the recombinant Vag8 proteins were eluted with Buffer B containing 300 mM imidazole. Imidazole in the Vag8 fraction was removed by dialysis against Buffer A. Protein concentrations were determined using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific). The recombinant proteins were subjected to SDS-PAGE followed by immunoblotting using anti-HAT-tag antibody (GenScript) and HRP-conjugated anti-rabbit IgG.
(Jackson ImmunoResearch). Target proteins were visualized with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore).

(ii) **ELISA-based binding assay.** Wells of 96-well microplates (ELISA Plate H; Sumitomo Bakelite) were coated with 0.1 ml of 10 nM C1 Inh (Complement C1 Inhibitor, Human; Calbiochem) at 4°C overnight, and then blocked with Dulbecco’s modified phosphate buffered saline (D-PBS) containing 10% skim milk at 37°C for 1 hour. Recombinant Vag8 proteins at the indicated concentrations were added to the wells and allowed to react at 37°C for 1 hour. The Vag8 proteins bound to C1 Inh were probed with a combination of anti-HAT-tag antibody, HRP-conjugated anti-rabbit IgG, and 0.1% TMBZ (Dojindo Laboratories). Each antibody reaction was carried out at 37°C for 1 hour. The substrate reaction was carried out at room temperature for 30 minutes and stopped by addition of 1.0 N H₂SO₄. After each step before the substrate reaction, the wells were washed with D-PBS containing 0.05% Tween-20. The optical density at 450 nm (OD₄₅₀) of each well was read with a Multi-Detection Microplate Reader (POWERSCAN HT; BioTek).

(iii) **Inhibitory effects of C1 Inh on PK activity.** Recombinant Vag8 proteins (1 µM) were incubated with 50 nM C1 Inh, and subsequently with 2 nM PK (Human Kallikrein; Enzyme Research Laboratories) at 37°C for 1 hour each. After incubation, the solution was mixed with the chromogenic substrate (S-2302, 0.5 mM; Chromogenix) in HEPES-NaHCO₃ buffer (9), and incubated at 37°C for 1 hour. The OD₄₀₅ value of each well, determined using a Multi-Detection Microplate Reader, was referred to as the protease activity of PK.
(iv) PK-C1 Inh complex formation assay. Recombinant Vag8 proteins (3 µM) were preincubated with 100 nM C1 Inh at 37°C for 1 hour in 27 mM HEPES buffer, pH 7.4, containing 191 mM NaCl and 0.68 mM EDTA (10). The resultant mixture was mixed with 100 nM PK, incubated at 37°C for 1 hour, and subjected to SDS-PAGE followed by immunoblotting using anti-C1 Inh antibody (Abcam) and HRP-conjugated anti-rabbit IgG as described above.

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References


autotransporter Vag8 binds human C1 esterase inhibitor and confers serum resistance.

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**Figure legends**

**FIG 1** Vag8 and its derivatives used in this study. (A) Schematic representations of Vag8 and each recombinant protein are shown. Vag8WT and truncated derivatives of Vag8 are listed with their names and corresponding amino–acid positions. Each recombinant protein has a HAT-tag at the N-terminus. (B) Immunoblotting of the recombinant Vag8WT and truncated derivatives.

**FIG 2** Abilities of Vag8 and its derivatives to interact with C1 Inh. (A) Binding of truncated Vag8 derivatives to C1 Inh analyzed by ELISA-based binding assay. The data are presented as means (n = 3) and SEM. (B) Cancellation of the inhibitory effects of C1 Inh on PK activity by truncated Vag8 derivatives. N/C is the negative control without PK, C1 Inh, and Vag8s. The data are presented as means (n = 3) and SEM. Statistical analyses were carried out by one-way analysis of variance and Tukey’s multiple comparison test using Prism 8 (GraphPad Software). *P < 0.01. (C) Immunoblotting of C1 Inh incubated with PK and truncated Vag8 derivatives. Note that C1 Inh bound covalently to PK to form a complex with larger molecular mass (upper arrowhead) (10).
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