1 **Translocational unfolding in clostridial binary iota toxin**

2 **complex**

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21

22 **Abstract**

23 **Protein translocation across the membrane is critical for microbial**
24 **pathogenesis and various cellular functions. Bacterial binary toxins such as**
25 **anthrax toxin are composed of enzyme components and a translocation channel,**
26 **which catalyses substrate unfolding and translocation. Here we report the**
27 **structures of the clostridial binary toxin (iota toxin) translocation channel Ib-pore**
28 **and its complex with ADP-ribosyltransferase Ia. The Ib-pore structure at atomic**
29 **resolution provides a similar structural framework as observed for the catalytic**
30 **ϕ -clamp of the anthrax protective antigen pore. However, the Ia-bound Ib-pore**
31 **structure showed a unique binding mode of Ia: one Ia binds to the Ib-pore, and the**
32 **Ia N-terminal domain interacts with Ib via two other Ib-pore bottlenecks with**
33 **multiple weak interactions. Furthermore, Ib-binding induces Ia N-terminal α -helix**
34 **tilting and partial unfolding, whereupon the unfolded N-terminus continues to the**
35 **ϕ -clamp gate. This study reveals the novel mechanism of N-terminal unfolding,**
36 **which is crucial for protein translocation.**

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38

39 **Introduction**

40 *Clostridium perfringens* iota toxin (iota), *C. difficile* toxin (CDT), *C. spiroforme* toxin
41 (CST), and *C. botulinum* C2 toxin belong to the family of binary toxins. It consists of
42 the enzymatic ‘A’ component with actin-specific ADP-ribosyltransferase and the ‘B’
43 component which binds to the host cell and functions as the translocation channel of
44 each enzymatic component (Ia,Ib; CDTa,CDTb; CSTa,CSTb; and C2I,C2II,
45 respectively). The ‘B’ component precursor is first cleaved off by a cellular protease,
46 then binds to the target cell via a receptor, forms a soluble oligomer termed the
47 ‘prepore’, and finally converts to a membrane-spanning pore in an acidified endosome.
48 The oligomer-receptor complex acts as a substrate docking platform that subsequently
49 translocates an enzymatic component into the cytosol from the acidified endosome.
50 Therein, the enzymatic component mono-ADP-ribosylates G-actin, inducing
51 cytoskeletal disarray and cell death. The enzymatic component consists of two domains:
52 the C-terminal domain with an actin-specific ADP-ribosyltransferase activity, and the
53 N-terminal domain, considered as a binding domain to the membrane-spanning
54 translocation component. The clostridial binary (iota) toxin consists of Ia and Ib¹⁻³.
55 However, although extensive structural and functional studies of Ia have been
56 conducted^{4,5}, little is known regarding the Ib translocation channel.

57 A similar binary toxin, anthrax toxin, constitutes a major virulence factor of *Bacillus*
58 *anthracis*, consisting of an enzymatic component (two enzymatic proteins, edema (EF)
59 and lethal (LF) factors) and a protein translocation channel (protective antigen (PA))⁶.
60 LF is a zinc-dependent protease that cleaves mitogen-activated protein kinase kinase;
61 the combination of PA and LF leads to the death of humans and animals. Cleavage of a

62 PA precursor triggers heptamerization or octamerization (prepore), and the low pH
 63 endosomal environment causes the oligomer to insert into the membrane by forming a
 64 transmembrane β -barrel (pore)⁷. PA heptameric prepore structure was revealed with and
 65 without anthrax toxin receptor^{8,9}. Furthermore, the LF-bound PA prepore complex
 66 structure (PA dimer with one LF N-terminal domain (LF_N)) was revealed by
 67 crystallography at 3.1 Å resolution. The biological unit structure (PA₈(LF_N)₄) was also
 68 deduced from the complex, revealing that the first N-terminal helix of LF binds on the
 69 PA dimer interface surface¹⁰.

70 Although PA pore instability has hampered its structural analysis, Jiang et al.
 71 clarified the PA heptameric pore structure by cryo-electron microscopy (cryo-EM) at
 72 2.9 Å resolution¹¹, revealing the catalytic ϕ -clamp and a long membrane-spanning
 73 channel¹². The ϕ -clamp is the narrowest passageway of the PA translocation channel, in
 74 which the seven phenylalanine-427 residues converge within the lumen, generating a
 75 radially symmetric solvent-exposed aromatic ring. Krantz et al. proposed that the
 76 ϕ -clamp serves a chaperone-like function, interacting with hydrophobic sequences
 77 presented by the protein substrate as it unfolds during translocation¹². Because
 78 translocation is driven by a transmembrane proton gradient, a Brownian ratchet model
 79 has been proposed, which depends on protonation and deprotonation of the
 80 translocating polypeptide acidic residues¹²⁻¹⁵. Furthermore, an allosteric
 81 helix-compression model regulated by the α -clamp in PA was recently proposed¹⁶,
 82 although it remains controversial whether this model is adequate.

83 To our knowledge, no high-resolution structure of the enzyme-bound translocation
 84 channel including the LF-bound PA-pore is available (although the PA heptamer pore

85 structure with three LF_N was reported at 17 Å resolution¹⁷), and pore structural
 86 information remains limited in PA¹¹ and other type Tc toxins^{18,19}. In clostridial binary
 87 toxin, membrane prepore and pore structures have not been described. To understand
 88 the protein translocation mechanism via the bacterial translocation channel, here we
 89 determined the structures of the Ib heptameric translocation channel without and with
 90 the enzymatic component Ia (Ib-pore and Ia-bound Ib-pore, respectively) by cryo-EM at
 91 atomic resolution, providing the novel structural pose of Ia just prior to translocation in
 92 the transmembrane spanning Ib-pore.

93

94 **Results**

95 **Ib oligomerization**

96 Ib oligomerization is rapidly induced at 37°C in Vero cells in a temperature but not pH
 97 dependent-manner^{20,21}. Conversely, *in vitro*, Ib oligomerizes poorly at 37°C and/or
 98 under acidic conditions following cleavage of the 20 kDa N-terminal propeptide. PA
 99 can oligomerize and form the PA-prepore upon propeptide cleavage, with the
 100 conformational change from prepore to pore being induced at low pH *in vitro*²²;
 101 however, this leads to rapid and irreversible aggregation^{11,23}. During cryo-EM sample
 102 preparation *in vitro*, we found that 10% ethanol could efficiently induce oligomerization
 103 upon N-terminal propeptide cleavage (Extended Data Fig. 1). Therefore, the sample was
 104 prepared by adding 10% ethanol and 0.03% lauryl maltose neopentyl glycol (LMNG)
 105 (Anatrace) following propeptide cleavage. Consequently, EM imaging showed that it
 106 led to the direct conversion of monomeric Ib to the pore, not the prepore. The obtained

107 Ib-pore was stable at neutral pH, allowing cryo-EM structural analysis of the Ib-pore
108 along with the Ia-bound Ib-pore.

109

110 Ib-pore and Ia-bound Ib-pore cryo-EM data

111 Two data sets were collected using Titan Krios (FEI). With the first, we revealed the
112 Ib-pore structure using 38,433 particles after classification of 299,491 extracted
113 particles; local resolution analysis by Resmap²⁴ showed the inner pore region at
114 approximately 2.5 Å resolution ([Fig. 1a](#), [Extended Table 1](#), [Extended Data Fig. 2](#)). As
115 described in Online Methods, the first data also included small amounts of Ia-bound
116 Ib-pore particles, which provided a 5.2 Å resolution map of the Ia-bound Ib-pore
117 ([Extended Data Fig. 2](#)).

118 Therefore, we generated another data set, raising the ratio of Ia. Two classes clearly
119 showed Ia density on the Ib-pore with long β-barrel stem and short β-barrel stem,
120 respectively. Therefore, using 135,359 particles for short stem class and 62,940 particles
121 for long stem class following classification of 871,264 extracted Ia-bound Ib-pore
122 particles, 3D refinement was performed after several additional classifications
123 ([Extended Data Fig. 3](#)). Finally, 2.8 Å map (Ia-bound Ib-pore with short stem) and 2.9
124 Å map (Ia-bound Ib-pore with long stem) were yielded.

125

126 Structure of the Ib-pore

127 The obtained cryo-EM map shows the Ib-pore having a funnel-like structure, lacking
128 the lower stem region (328–365), suggesting that the bottom half of the stem had not yet
129 formed. Thus, the Ib-pore structure contains a stem of 40 Å length (90 Å at full length,
130 as noted for the Ia-bound Ib-pore), with the stem diameter the same as that of PA (>15

131 Å) (Fig. 2a-c; Extended Data Fig. 4a, 5a). The Ib-pore consists of four domains, 1'
 132 (domain 1 without the propeptide), 2, 3, and 4, as with PA. The main pore body
 133 comprises domain 2, which consists of two parts designated as 2c (residues 296–311
 134 and 381–512) and 2s (residues 312–380) (Fig. 2a, c). Domain 2s is an extended β
 135 -hairpin, seven copies of which assemble to form a membrane-spanning 14-stranded
 136 β -barrel. Domain 3 is located at an intermediate position between domains 1' and 2c.
 137 The cryo-EM density of domain 4, the receptor-binding domain, is weak and has the
 138 lowest resolution among all four domains (Extended Data Fig. 2), likely owing to its
 139 flexibility resulting from minimal contact with the other domains. The overall structure
 140 of the Ib-pore is similar to that of the PA-pore (Extended Data Fig. 5a). Specifically, the
 141 funnel structure consisting of domains 1', 2, and 3 shows high similarity with that of the
 142 PA-pore. Domains 1 and 2 of PA share 41 and 40% sequence identity with the
 143 corresponding regions of Ib (Extended Data Fig. 6). The weak map density of domain 4
 144 is also reported for the PA-pore¹¹. In Ib, the relative position of this density differs from
 145 that of PA-pore domain 4 (Extended Data Fig. 5a). As domain 4 is a receptor binding
 146 domain, the sequence identity shows < 10% identity with PA, with the size also
 147 differing (Ib domain 4 is twice as large as that of PA).

148 The cryo-EM density of the inner side of the funnel was analysed at high resolution;
 149 thus, the side chain is clearly visible (Extended Data Fig. 4b). The narrowest clamp is
 150 formed by seven F454s from seven protomers with a diameter of 6 Å, termed the
 151 ϕ -clamp in PA (Fig. 2e). Two additional bottlenecks exist in the cis-side: Ca-edge
 152 (216–224), an N-terminal Ca binding site, and NSQ-loop (490–492), with 45 Å and 23
 153 Å diameters, respectively (Fig. 2d). Ca-edge, which is a unique di-calcium binding site

([Extended Data Fig. 5b](#)), and NSQ-loop are the most important regions for Ia-binding as described later. We then divided the inner pore surface as layer I, II, III, IV, and V from the cis- to trans-side ([Fig. 2c](#)). Layer I represents the broadest area of the funnel from the Ca-edge to NSQ-loop. Layer II is from the NSQ-loop to E498-loop. Layer III is from the E498-loop to ϕ -clamp. Layer IV is from the ϕ -clamp to H313 in the trans-side. We designated the stem region as layer V. The long stem with a β -barrel is created by an amphipathic flexible loop (E312–I380) of the prepore.

The enzymatic component Ia (ADP-ribosyltransferase) is translocated from the cis (layer I, II, and III) to the trans-side (layer IV and V). Although the sequence is not well-conserved on the inner surface between the Ib-pore and PA-pore, structural similarities exist and the negative (pH 7) and positive (pH 5.5) surface potential in the cis side (Layer II and III) are maintained in both channels ([Extended Data Fig. 5c](#)). In PA, N422 and D425 (422–NAQDDFSST–430) were noted as constituting an essential pH sensor to lead the conversion from prepore to pore; in Ib, these two residues are conserved. Notably, H313 locates on the inner surface of the upper stem, which appears to have an important function for translocation ([Fig. 2c](#)). Moreover, numerous Ser and Thr residues exist in the inner surface stem region, which may have vital functions as with PA although the residue positions are not conserved between Ib and PA (stem region Ser/Thr content of 56% each in Ib and Pa) ([Extended Data Fig. 5c](#)).

173

174 **Structure of the Ia-bound Ib-pore**

175 Two separated classes, Ia-bound Ib-pore with short and long stem, were obtained from
176 the second data set by C1 data analysis ([Extended Data Fig. 3](#)). In both structures, Ia sits

177 on the cis-side of the Ib-pore with the same binding mode (Fig. 1b-c and 3a-c; Extended
178 Data Fig. 4a). Notably, the Ia stoichiometry and binding mode differed from those of
179 the LF-bound PA-pore (PA:LF = 8:4 or 7:3). Clearly, one Ia binds to the Ib-pore
180 heptamer. Half of the Ia N-terminal domain is buried in the Ib-pore through several
181 interactions with interface 1650 Å². They primarily interact asymmetrically with
182 Ca-edges and NSQ-loops. Specifically, five Ca-edges in subunit C-G contribute to Ia
183 binding, with seven NSQ-loops in subunit A-G supporting the Ia N-terminal domain
184 (Fig. 3d-g; Extended Data Fig. 7).

185 No large Ia-induced structural change occurs in the Ib-pore between the apo-Ib-pore
186 and Ia-bound Ib-pore; only two NSQ-loops (E and F subunits) exhibit asymmetric
187 positional change (Fig. 3f). Moreover, no large structural change occurs in almost the
188 entire Ia molecule except the N-terminal (1–44) region between apo-Ia and the Ia-bound
189 Ib-pore (Fig. 3h). Within the seven NSQ-loop interaction, one (F subunit) pushes
190 residues 29–32 in the N-terminal α-helices (Ia), causing the apparent tilt and partial
191 unfolding of the N-terminal α-helix (Fig. 3h; Extended Data Fig. 7). Thus, the
192 N-terminal region (1–17) in Ia unfolds in the Ib-pore because it is too large to fit in the
193 NSQ-loop bottleneck, then continues to the Ib-pore φ-clamp gate (Fig. 3h, i). It appears
194 that the Ia molecule floats from the Ib protomers via NSQ-loops, thus providing free
195 inner space to accommodate the Ia N-terminal region. In summary, the main
196 interactions are caused by several asymmetrical Ca-edges and NSQ-loops. No specific
197 strong interaction exists between Ia and the Ib-pore, suggesting that this may constitute
198 an essential feature for the translocation channel and substrate protein, with the weak
199 interactions affording efficient translocation.

200 Although detailed reports regarding Ia–Ib binding are lacking, an earlier study
 201 showed that the N-terminal domain (residue 216–321 in domain 1') of Ib is essential for
 202 Ia docking²⁵. This cryo-EM study of the Ia-bound Ib-pore provides precise information
 203 regarding Ia and Ib-pore interactions, showing the Ca-edge of domain 1' along with the
 204 NSQ-loop of domain 2 as essential for binding. It was also reported that Ib lacking just
 205 the first N-terminal 27 residues did not facilitate Ia entry²⁵. This is because the
 206 N-terminal 27 residues form the Ib-pore Ca²⁺ binding site (Ca-edge) ([Extended Data Fig.](#)
 207 [5b](#)). Moreover, residues 129–257 were proposed as the minimal Ia fragment for
 208 translocation²⁶. The present Ia-bound Ib-structure showed that a native ordered structure
 209 of the Ia N-terminal domain (45–210) is necessary for the stacking via NSQ-loops.

210 We next compared previous mutational results of Ib with the present Ib-pore
 211 structure. F454A led to loss of cytotoxicity and markedly increased single-channel
 212 conductance, suggesting that the ϕ -clamp is highly conserved and crucial for binary
 213 toxin activity²⁷. Several mutations within the amphipathic β -strand forming the stem
 214 affected pore formation, single-channel conductance, and ion selectivity (S339E–S341E,
 215 Q345H, and N346E)²⁷. Based on the structure, S339, S341, and N346 are located on the
 216 inner stem surface, whereas Q345 is found on the stem tip. Ser and Thr residues on the
 217 inner surface are likely essential for translocation.

218

219 Discussion

220 The available cryo-EM structure of LF_N-bound PA-pore (PA₇(LF_N)₃) is at low
 221 resolution; nevertheless, the same binding mode as in the LF_N-bound PA-prepore is
 222 assumed^{10,17}. The first α -helix and β -strand of LF_N unfold and dock into the deep

223 amphipathic cleft on the octamer surface, termed the ‘ α -clamp’ (Fig. 3j). Thus, the main
 224 interactions are formed by the N-terminal helix binding to the PA-prepore α -clamp.
 225 Although similar structural feature and electrostatic potential exist for Ib-pore
 226 translocation channel, significant differences exist between Ia and LF binding. For LF,
 227 the N-terminal α -helix binds to the PA-pore α -clamp, with the following 26 N-terminal
 228 residues being invisible owing to their flexibility. This flexibility is also observed in the
 229 apo-structure, suggesting that the N-terminal region is intrinsically flexible (Fig. 3k).
 230 Conversely, for Ia, binding to the channel causes Ia N-terminal unfolding. This indicates
 231 that the Ib-pore serves as an unfolding chaperone for substrate translocation even at
 232 neutral pH. As the Ib α -clamp site does not function as an α -clamp, the Ib-pore rather
 233 uses a novel mechanism for Ia N-terminal region unfolding: (1) a large portion of the
 234 N-terminal domain of the Ia structure lies in the Ib-pore, and (2) the interaction induces
 235 Ia N-terminal helix tilting and partial unfolding (Fig. 3h). Thus, the Ib-induced
 236 disordered region (1–AFIERPEDFLKD–12) followed by unfolded N-terminal helix
 237 (13–KENAI–17) directly continues to the ϕ -clamp (Fig. 3i).

238 The Ia N-terminal region contains numerous hydrophobic along with both positive
 239 and negative residues. Ia and LF share no sequence similarity, suggesting that the
 240 characteristic residue assortment (positive, negative, and hydrophobic) is essential for
 241 translocation. Constructs lacking both negative and positive charges in the unstructured
 242 region of LF_N, composed of only Gly, Ser, and Thr, translocate more slowly and
 243 independently of the Δ pH, indicating that a balance of acidic- and basic-charged
 244 residues is required for efficient translocation with Δ pH²⁸. Furthermore, in endosomes,
 245 as the phenylalanine clamp could be considered as a barrier of pH difference, the

246 cis-side (Layer II and III) electrostatic potential is positive (pH 5.5), whereas the
 247 trans-side (IV) electrostatic potential is negative (pH 7.0) ([Extended Data Fig. 5c](#)). The
 248 electrostatic potential difference represents a common aspect in Ib-pore and PA-pore
 249 and is likely important for translocation. It is also noted that electrostatic repulsion
 250 between pore (NSQ-loop in subunit E) and substrate protein (Arg26) seems to be
 251 important for efficient translocation ([Extended Data Fig. 7b](#)). Furthermore we consider
 252 that the destabilization of Ca-edge at endosomal acidic pH is also key to reduce the
 253 interaction, leading to more efficient translocation.

254 Despite the differences of binding stoichiometry and binding mode of Ia/Ib and
 255 LF/PA, the unfolded Ia and LF N-terminals are accommodated in electrostatically
 256 charged cis-side pore, then led to the ϕ -clamp. Therefore, we consider that the first entry
 257 event, in which the tip of the unfolded N-terminus enters into the gate of the
 258 hydrophobic ϕ -clamp, is significant for translocation, similar to threading through a
 259 needle. For Ia, the unfolded N-terminal region becomes freely accessible to the ϕ -clamp
 260 in the space of Layer III under the NSQ-loop. Thus, Ia N-terminal movement in the
 261 limited space is beneficial compared with LF-bound PA with large open space, allowing
 262 the hydrophobic tip to readily reach the hydrophobic ϕ -clamp gate. This first event
 263 appears necessary for an extended-chain Brownian ratchet model.

264 Two ϕ -clamp configuration states (clamped and unclamped dilated states) have been
 265 proposed that are allosterically regulated by the α -clamp^{16,29}. The allosteric
 266 helix-compression model was proposed as more favourable than the extended-chain
 267 Brownian ratchet model. This model explains that the successive α -helix formation
 268 induced by the α -clamp is essential for substrate translocation and that newly formed

269 α -helices pass through the dilated ϕ -clamp, leading to produce more power stroke.
 270 However, structures of both the PA-pore at acidic pH and Ib-pore at neutral pH show
 271 that the ϕ -clamp forms the same configuration as in the closed (clamped empty) state.
 272 The dilated (unclamped empty) state structure has not yet known; moreover, whether
 273 the dilated state exists and the allosteric helix-compression model is generalizable
 274 remain controversial^{30,31}. In Ib and C2II, the dilated state has not been observed by
 275 electrophysiological study^{32,33} or in the present cryo-EM studies. With regard to its
 276 structure, as seven phenylalanines are stacked in the ϕ -clamp structure, change to a
 277 more dilated conformation is unlikely. In addition to these, Ib doesn't use the α -clamp for
 278 α -helices binding. Together, these observations indicate that the Ib translocation likely
 279 occurs via a static ϕ -clamp pore, suggesting the extended-chain translocation of the
 280 unfolded N-terminal substrate (Fig. 4). The presented binary toxin complex structure
 281 and the mechanism of unfolded N-terminal substrate translocation should be conserved
 282 in other *C. difficile*, *C. spiroforme*, and *C. botulinum* binary toxins. Notably, our study
 283 provides structural clues to develop inhibitors of these binary toxins, especially CDT
 284 from human opportunistic pathogen *C. difficile*³⁴⁻³⁶ or iota-like toxin (CPILE/BEC) in
 285 human food poisoning outbreaks³⁷⁻³⁹.

286 At neutral pH, only a minor conductance decrease was observed upon Ia addition to
 287 the membrane cis-side³². However, at pH 5.6, cis-side conductance decreased to
 288 30–40% of the open configuration conductance³². Ia-mediated Ib blockage occurred
 289 only at pH 5.6, suggesting the translocation occurs only at acidic pH. In our study, we
 290 revealed the Ia-bound Ib-pore structure at neutral pH. The disordered Ia N-terminal

291 region tip reaches the ϕ -clamp (Fig. 3i); however, it may not be entirely blocked but still
 292 fluctuate in the pore.

293 In summary, the present structure captured the Ia-bound Ib-pore just before
 294 translocation: The Ia N-terminal region exhibited Ib-induced unfolding. In future studies,
 295 using the stable Ib-pore, we expect to capture the translocation state of Ia in the Ib-pore
 296 under acidic conditions.

297

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415 **Author contributions**

416 T.Yamada, T.Yoshida, A.K., and H.T. participated in research design and data analyses;
417 T.Yamada prepared the Ib-pore and Ia-bound Ib-pore for cryoEM; T.Yamada, A.K.,
418 K.M., and K.I. performed cryoEM data acquisition and image processing; T.Yoshida
419 performed the atomic model building, structure refinement and analyses; all author
420 contributed to writing the manuscript and H.T. supervise the project.
421

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425

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428 **Methods**

429 **Ib and Ia expression and purification.** The *iota toxin b (Ib)* gene was cloned into
 430 pGEX4T-1 without a signal peptide, and Ib was overexpressed in *Escherichia coli*
 431 Origami. The transformant was cultured in super broth medium containing ampicillin
 432 (50 µg/ml), tetracycline (12.5 µg/ml), and kanamycin (15 µg/ml) at 37°C until OD₆₀₀ of
 433 0.6, then isopropyl β-D-1-thiogalactopyranoside (final 1 mM) was added followed by
 434 culturing at 23°C for 16 h. The harvested cells were resuspended in lysis buffer
 435 containing 20 mM Tris pH 8.0, 150 mM NaCl, 2 mM CaCl₂, and 5 mM dithiothreitol,
 436 and disrupted by sonication. After centrifugation at 180,000 × g for 40 min, the
 437 supernatant was loaded onto a Glutathione Sepharose 4B resin (GE Healthcare) column.
 438 After washing the column with lysis buffer, the bound protein was eluted by a buffer
 439 containing 20 mM Tris pH 8.0, 150 mM NaCl, and 10 mM reduced glutathione. The
 440 eluted fractions were concentrated to 19 mg/ml and the buffer was exchanged to 20 mM
 441 Tris pH 8.0, 50 mM NaCl, 2.5 mM CaCl₂.

442 The *iota toxin a (Ia)* gene was cloned into pET-15b or pET-21b to produce Ia with
 443 N-terminal or C-terminal His-tag, respectively. Then, Ia was overexpressed in *E. coli*
 444 BL21 Star (DE3). The transformant was cultured in super broth medium containing
 445 ampicillin (50 µg/ml) at 37°C until OD₆₀₀ became 1.5 and then isopropyl
 446 β-D-1-thiogalactopyranoside was added (final 0.5 mM) followed by culturing at 37°C
 447 for 16 h. The harvested cells producing each Ia were individually resuspended in lysis
 448 buffer containing 20 mM Tris pH 8.0, 300 mM NaCl, and 20 mM imidazole, and
 449 disrupted by sonication. After centrifugation at 180,000 × g for 40 min, the supernatant
 450 was loaded onto a Ni-NTA agarose column. After washing the column with lysis buffer,

451 the bound protein was eluted by a buffer containing 20 mM Tris pH 8.0, 300 mM NaCl,
452 and 500 mM imidazole. The eluted fractions were concentrated and the buffer
453 exchanged to that containing 20 mM Tris pH 8.0, 2 mM CaCl₂ to load onto an HiTrap Q
454 HP 5 ml column (GE Healthcare). After anion exchange purification, Ia with C-terminal
455 His tag was concentrated to 2.63 mg/ml, and the buffer was exchanged to 10 mM Tris
456 pH 8.0 and 100 mM NaCl. Alternatively, Ia with N-terminal His-tag was incubated with
457 0.001 (unit/μg Ia) thrombin for 16 h at room temperature. Ia, in which the N-terminal
458 His-tag was cleaved, was loaded onto a Ni-NTA agarose column, then the flow-through
459 and wash fractions were collected. Collected fractions were concentrated to 22 mg/ml
460 and buffer was exchanged to 10 mM Tris pH 8.0 and 100 mM NaCl using an Amicon
461 filter.

462 **Sample preparation for the first data set.** Ib oligomerizes poorly at neutral and acidic
463 pH *in vitro* after cleavage of the pre-sequence with α-chymotrypsin. Ib (30 mg) was
464 treated with 30 μg α-chymotrypsin for 1 h at room temperature. This reaction was
465 terminated by adding phenylmethylsulfonyl fluoride (PMSF) (final 1 mM). Then, Ia
466 with C-terminal His-tag was added to the Ib solution with three-fold molar excess and
467 incubated 1 h at 37°C. The solution was loaded onto a Ni-NTA agarose column. We
468 expected that the elution fraction includes Ia-bound Ib oligomer, but it was failed
469 because they didn't coexist in high concentration of imidazole. From oligomerization
470 screening, we found that ethanol induced oligomerization of Ib efficiently after cleavage
471 of pre-sequence incubating with α-chymotrypsin ([Extended Data Fig. 1a](#)). Accordingly,
472 we changed to use the ethanol oligomerization to apply the flow-through fraction
473 including Ib. Ethanol (final 10%) and LMNG (final 0.03%) was added to 1 mg Ib in the

474 flow-through fraction and incubated at 37°C for 1 hour. In order to separate Ib oligomer
475 from other small proteins, the solution was loaded onto 10.5 ml of Glycerol gradient
476 bed (10–30% Glycerol, 50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM CaCl₂, 0.03%
477 LMNG). After ultracentrifuge at $230,139 \times g$ for 16 hours, fractions were collected by
478 250 µl from bottom. Fractions showing high molecular mass by sodium dodecyl
479 sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were collected and buffer
480 was exchanged to 10 mM HEPES pH 7.5, 1 mM CaCl₂, and 0.01% LMNG and
481 concentrated to 1.9 mg/ml. This sample included Ib-pore and a small amount of Ia
482 ([Extended Data Fig. 1b, c](#)).

483 **Sample preparation for the second data set.** During sample preparation for the first
484 data set, binding between Ia and the Ib-pore occurred and the LMNG concentration was
485 too high for cryo-EM data collection. To solve these problems, Ib was purified with a
486 smaller concentration of LMNG and Ia was added following Ib-pore purification. Ib (21
487 mg) was cleaved by 1 µg α-chymotrypsin for 1 h at room temperature. This reaction
488 was terminated by adding PMSF (final 1 mM). The solution was then treated with 10%
489 ethanol and 0.03% LMNG for 1 h at 37°C. To separate Ib oligomer from other small
490 proteins, the solution was loaded onto 10.5 ml of Glycerol gradient bed (10–30%
491 Glycerol, 50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM CaCl₂, 0.003% LMNG). After
492 ultracentrifugation at $230,139 \times g$ for 16 h, 250 µl fractions were collected from the
493 bottom. Fractions showing high molecular mass by SDS-PAGE were collected and the
494 buffer was exchanged to 10 mM HEPES pH 7.5, 1 mM CaCl₂, and 0.003% LMNG.
495 Purified Ib oligomer was concentrated to 2.4 mg/ml, then Ia without His-tag was added
496 with three-fold molar excess at the final step ([Extended Data Fig. 1d, e](#)).

497 Cryo-EM imaging of the Ib-pore for the first data set. Sample vitrification was
 498 performed using a semi-automated vitrification device (Vitrobot Mark IV, Fisher
 499 Scientific, Eindhoven, The Netherlands). A 2.6 μ l aliquot of sample solution at a
 500 concentration of 0.38 mg/ml (1/5 dilution of the sample) was applied to
 501 glow-discharged Quantifoil R1.2/1.3 in the Vitrobot at 100% humidity. The grid was
 502 then automatically blotted once from both sides with filter paper for 4.5 s blot time. The
 503 grid was then plunged into liquid ethane with no delay time. Cryo-EM imaging was
 504 performed using a Titan Krios (Fisher Scientific) operating at 300 kV acceleration
 505 voltage and equipped with a Cs corrector (CEOS, GmbH) and a direct electron detector
 506 Falcon 3 (counted mode) (Fisher Scientific) in automated data collection mode at a
 507 calibrated magnification of 1.13 $\text{\AA}/\text{pixel}$ (magnification $\times 59,000$) and dose of 50 $\text{e}/\text{\AA}^2$
 508 (or 0.46 $\text{e}/\text{\AA}^2$ per frame) with total 84.09 s exposure time. The data were automatically
 509 collected using EPU software with a defocus range of -0.8 to -2.5 μm and were
 510 fractionated 108 movie frames.

511 Image processing of the Ib-pore for the first data set. A total of 2,120 images were
 512 collected in the first data set. The movie frames were subsequently aligned to correct for
 513 beam-induced movement and drift using MOTIONCORR2⁴⁰, and contrast transfer
 514 function (CTF) were estimated using CTFFIND4⁴¹. A total of 299,491 particle images
 515 were automatically picked using Gautomatch (<http://www.mrc-lmb.cam.ac.uk/kzhang/>)
 516 and several rounds of 2D classification and 3D classification were performed using
 517 RELION-3.0⁴². The best among the 3D classes in which clearly showed 7-fold
 518 rotational symmetry in Ib-pore were subjected to 3D refinement with C7 symmetry. The
 519 3D-refined structure was further CTF refined using the per-particle defocus and

520 Bayesian polishing, which improved the resolution to 2.9 Å and a B-factor of -46 Å^2
 521 without the substrate Ia ([Extended Data Fig. 2a-e](#)).

522 **Image processing of the Ia-bound Ib-pore for the first data set.** While the 3D class
 523 reconstruction proceeded in Ib-pore analysis, we identified another class that showed Ia
 524 density on the Ib-pore (1,735 particles). The class was subjected to 3D refinement and
 525 used as a template for a second 3D classification with 154,378 particles. Classes that
 526 exhibited density on the Ib pore were selected for processing using 3D refinement.

527 Around the Ib pore membrane spanning stem (outside of the stem), some blurred
 528 density was observed that appeared irregularly in each class; therefore, it was subtracted
 529 to increase the efficiency of classification. Subtracted particles were subjected to a third
 530 3D classification, and then classes that contained 15,890 particles with strong density on
 531 the Ib pore were subjected to 3D refinement initially without and then with a solvent
 532 mask. Finally, an Ia-bound Ib map was generated at 5.2 Å resolution ([Extended Data](#)
 533 [Fig. 2f-j](#)). This result prompted us to collect a second data set using the sample while
 534 raising the ratio of Ia.

535 **Cryo-EM imaging of the Ia-bound Ib-pore for the second data set.** A 2.6 µl aliquot
 536 of sample solution at a concentration of 0.48 mg/ml (1/5 dilution of the sample) was
 537 applied to glow-discharged Quantifoil R1.2/1.3. Other procedures including sample
 538 vitrification, blotting, freezing, and Cryo-EM imaging were the same as described for
 539 the first data set.

540 **Image processing of the Ia-bound Ib-pore for the second data set.** Image processing
 541 was performed as described for the first data set unless otherwise stated. A total of
 542 2,151 images were collected in the second data set. The movie frames were
 543 subsequently aligned to correct for beam-induced movement and drift using

544 MOTIONCORR⁴⁰, and CTF were estimated using GCTF⁴¹. A total of 871,264 particle
 545 images were automatically picked using Gautomatch and several rounds of 2D
 546 classification were performed using RELION-3.0⁴². A total of 335,767 particles in the
 547 best class were subjected to 3D refinement, per-particle CTF refinement and Bayesian
 548 polishing. The polished particles were subjected to 3D classification using 3D
 549 refinement structure as the reference and divided into eight classes. The two classes
 550 clearly showed Ia density on the Ib-pore with long stem and short stem, respectively.
 551 These two classes were subjected to 3D refinement, per-particle CTF refinement and
 552 Bayesian polishing. The resulting 3D structure was further subjected to no-align 3D
 553 classification using a mask covering Ia. The final 3D refinement and postprocessing of
 554 two classes yielded maps with global resolution of 2.91 Å and B factor of -29 Å²
 555 (Ia-bound Ib-pore with long stem) and 2.80 Å and B factor of -10 Å² (Ia-bound Ib-pore
 556 with short stem), according to 0.143 criterion of the FSC ([Extended Data Fig. 3](#)).
 557 **Model building and refinement.** An Ib-pore model with short stem was built using the
 558 first data set. An initial rigid-body fit of PA structure (PDB ID: 3J9C) was applied into
 559 the cryo-EM density map using UCSF Chimera⁴³. The Ib-pore model was then
 560 manually built by iterative rounds of model modification in COOT⁴⁴ and refinement
 561 using PHENIX Real Space Refinement with secondary structure restraint⁴⁵. Model
 562 building of Ib Domain 4 was not carried out because of the low resolution and lack of
 563 available crystal structure.

564 An Ia-bound Ib-pore model with short stem was built using the cryo-EM density
 565 map with partial stem from the second data set. Following initial model building and
 566 rigid-body fit using the Ib-pore model from the first data and the crystallographic

567 structure of Ia (PDB ID: 1GIQ), they were manually modified and refined by iterative
568 rounds of COOT and PHENIX as for model building of the Ib-pore with short stem.

569 Furthermore, an Ia-bound Ib-pore model with long stem was built using the
570 cryo-EM density map with intact stem from the second data set. Following rigid-body
571 fit of the Ib-pore model with short stem, the intact long stem was manually built using
572 COOT and they were modified and refined by iterative rounds of COOT and PHENIX.
573 Although the density of Ia (C-terminal domain) was insufficient for *de novo* model
574 building, the cryo-EM density maps with short and long stem showed that the Ia and Ib
575 conformations of the two maps were the same. Therefore, rigid-body fitting of the Ia
576 model from the Ia-bound Ib-pore with long stem was finally carried out using UCSF
577 Chimera. Then, the final Ia-bound Ib-pore with long stem model was refined using
578 PHENIX Real Space Refinement with secondary structure restraint.

579 In the manuscript, we discussed Ia binding using the Ia-bound Ib-pore model with
580 short stem because the cryo-EM density of Ia with short stem was clearer than that with
581 long stem. The structures with long and short stem are the same except for stem length.
582 The difference of cryo-EM density of Ia was likely attributed to the difference of
583 particle number (short:135,359 and long: 62,940).

584 All figures were prepared using PyMOL (<https://pymol.org/2/>), UCSF Chimera, and
585 UCSF ChimeraX⁴⁶.

586

587 **Data availability**

588 Cryo-EM maps and coordinates were deposited in the Electron Microscopy Data Bank
589 and Protein Data Bank with the accession codes EMDB-0721 and PDB 6KLX for the

590 Ib-pore, EMDB-0713 and PDB 6KLO for the Ia-bound Ib-pore with short stem, and
591 EMDB-0720 and PDB 6KLW for the Ia-bound Ib-pore with long stem.

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Figure 1 | Cryo-EM density maps.

a, Ib-pore with short stem. **b**, Ia-bound Ib-pore with short stem. **c**, Ia-bound Ib-pore with long stem. Ib protomers are shown in different colours.

Figure 2 | Atomic model of the Ib-pore.

a, Ib Domain structure. **b**, Overall structure of the Ib-pore with short stem. The domain IV model was not built. **c**, Ib-pore layer structure. Five layers are shown in a protomer cartoon model colour-coded as in **a** along with the cut-away surface electrostatic potential at pH 7.0. The amino acid residues forming the boundary between adjacent layers are shown as a sphere model. **d**, Three bottlenecks of the Ib-pore. Ca-edge (216–224), NSQ-loop (490–492), and φ-clamp (454). Surface model is shown. Diameters of bottlenecks are shown in parentheses. **e**, Top view of the f-clamp with cryo-EM density map.

Figure 3 | Atomic model of the Ia-bound Ib-pore.

a, Ia domain structure. **b,c** Ia-bound Ib-pore overall structures with short and long stems. **d**, Ia, bottom view. **e**, Cut-away surface electrostatic potential at pH 7.0. **f**, Top views. For clarity, 1–210 (left) and 1–44 (center) regions of Ia are shown. Ia is not shown (right). **g**, Cut-away view. **h**, Unfolding of Ia N-terminus. Ib-bound Ia and Ia crystal structure are superimposed. **i**, Extra map leading from the N-terminal helix to ϕ -clamp. Map within 3 Å of the Ib model is subtracted from the original map. **j**, LF-bound PA-prepore structure. **k**, PA-bound LF and LF crystal structure superposition.

Figure 4 | Translocation model of Ia via Ib-pore.

Ia (black) and Ib-pore (grey). Arrows indicate possible Ia movement direction. **a**, Ia and Ib-pore before complexation. **b**, Ia-bound Ib-pore complex solved herein by single particle analysis. Ib-pore binding induces tilts and partial unfolding of the first N-terminal Ia α -helix. **c**, Endosomal acidic conditions facilitate unfolded N-terminal tip entry into the Ib-pore ϕ -clamp followed by extended-chain Brownian ratchet model-mediated translocation. **d**, Expected mechanism to prevent Ia retro-translocation by α -helix formation in the stem at neutral pH in the trans-side.