Structure–function analyses of a pertussis-like toxin from pathogenic *Escherichia coli* reveal a distinct mechanism of inhibition of trimeric G-proteins

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Pertussis-like toxins are secreted by several bacterial pathogens during infection. They belong to the AB2 virulence factors, which bind to glycans on host cell membranes for internalization. Host cell recognition and internalization are mediated by toxin B subunits sharing a unique pentameric ring-like assembly. Although the role of pertussis toxin in whooping cough is well-established, pertussis-like toxins produced by other bacteria are less studied, and their mechanisms of action are unclear. Here, we report that some extra-intestinal *Escherichia coli* pathogens (i.e. those that reside in the gut but can spread to other bodily locations) encode a pertussis-like toxin that inhibits mammalian cell growth *in vitro*. We found that this protein, EcPlt, is related to toxins produced by both nontyphoidal and typhoidal *Salmonella* serovars. Pertussis-like toxins are secreted as disulfide-bonded heteroheexamers in which the catalytic ADP-ribosyltransferase subunit is activated when exposed to the reducing environment in mammalian cells. We found here that the reduced EcPlt exhibits large structural rearrangements associated with its activation. We noted that inhibitory residues tethered within the NAD+-binding site by an intramolecular disulfide in the oxidized state dissociate upon the reduction and enable loop restructuring to form the nucleotide-binding site. Surprisingly, although pertussis toxin targets a cysteine residue within the α subunit of inhibitory trimeric G-proteins, we observed that activated EcPlt toxin modifies a proximal lysine/asparagine residue instead. In conclusion, our results reveal the molecular mechanism underpinning activation of pertussis-like toxins, and we also identified differences in host target specificity.

Pathogenic *Escherichia coli* strains preferentially inhabit different sites within their hosts, with enteric or extra-intestinal niches presenting distinctly different challenges. Infection by enteric or diarrheagenic *E. coli* can result in gastroenteritis, but it seldom spreads beyond the intestinal tract except in immunocompromised individuals (1, 2). In contrast, extra-intestinal *E. coli* (ExPEC)7 strains such as uropathogenic *E. coli* (UPEC) or neonatal meningitis *E. coli* can reside passively within the gut until conditions permit their expansion into the urinary tract, blood, or nervous system where they may potentially cause life-threatening disease (3). To do so, ExPEC strains express a range of virulence factors, often encoded on mobile genetic elements, including AB2 toxins. Such phage-encoded toxins are secreted by several major bacterial pathogens, including enterotoxigenic *E. coli* (55) and enterohemorrhagic *E. coli* (4); *Vibrio cholerae*

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This article was selected as one of our Editors’ Picks. This article contains supplemental Methods, Movie S1, Table S1, and Figs. S1–S6.

The atomic coordinates and structure factors (codes 4Z9C and 4Z9D) have been deposited in the Protein Data Bank (http://wwwpdb.org/).

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7 The abbreviations used are: ExPEC, extra-intestinal *E. coli*; ART, ADP-ribosyltransferase; GPCR, G-protein-coupled receptor; Plt, pertussis-like; TCEP, tris(2-carboxyethyl)phosphine; PT, pertussis toxin; HBS, HEPES-buffered saline; UPEC, uropathogenic *E. coli*.
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(5), Shigella (6), Salmonella (7), and Bordetella pertussis (8, 9). Modern sequencing techniques frequently identify novel AB₅ toxins related to those previously studied, but their conservation at the level of function remains to be determined.

AB₅ virulence factors bind to glycans present on the surface of eukaryotic host cell membranes resulting in their internalization. Once inside the host cell, the enzymatic A subunits are released allowing them to disrupt host biochemistry and physiology. Host cell recognition and internalization are mediated by the toxin B subunits that share a unique pentameric ring-like assembly. This juxtaposes with the A subunit cargos that are class-specific and structurally divergent, and their activation inside mammalian cells occurs through distinct intracellular detection and release mechanisms (10). Five AB₅ toxin families currently exist: the enzymatic components of the subtilase cytotoxin (11) and EcxBAB toxin (12) are proteases, whereas those of the Shiga group (6) are ribosome inhibitors; cholera and heat-labile enterotoxins (4, 5) carry ADP-ribosyltransferases, as do the related pertussis (8, 9) and typhoid toxin (7) proteins.

Toxin ADP-ribosyltransferases (ARTs) hydrolyze the nicotinamide group from NAD⁺ and transfer the ADP-ribose moiety onto specific host proteins. Pertussis toxin specifically targets inhibitory trimeric G-proteins by modifying a conserved cysteine located four residues from the C termini of the Gα subunit (13). This modification renders the Gαₙ/i/o subunits unable to associate with their cognate G-protein-coupled receptors (GPCRs) thus modulating the host’s immune response. A series of related pertussis-like (Plt) toxins exist within the genomes of pathogenic bacteria, including strains of E. coli, Salmonella, and Yersinia. The bacterial strains harboring such virulence factors are diverse, and their evolutionary relationship is complicated due to the spread of these genes on mobile genetic elements. Proteins within the Plt family include the atypical archetype member whose glycan-binding B subunits have expanded and diverged within the B. pertussis genome to form four separate genes yielding a pseudopentameric glycan-binding platform. In contrast, other pertussis-like toxins display a homopentameric glycan-binding stoichiometry. All Plt proteins nonetheless carry a conserved catalytic A subunit.

In the secreted state, the enzymatic ART domain of pertussis-like toxins lies atop the five glycan-binding subunits allowing its C terminus to thread through a U-shaped NAD⁺-binding cleft before plunging into the pore of the B subunit pentamer (7, 9). When these C-terminal residues are truncated from pertussis toxin, its ART domain is constitutively active in vitro but is unable to associate with its pseudopentamer or enter cells (14). Enzymatic activation in vivo requires proteolytic separation of the inhibitory C terminus and reduction of a connecting disulfide. Although the molecular mechanisms underlying an alternative activation mechanism of cholera toxin are understood (15), the changes that occur following activation of a pertussis-like protein have not previously been characterized.

Although the association of pertussis toxin with whooping cough is well-established, orthologous pertussis-like toxins present within other pathogenic bacterial infections are less studied, and their mechanism(s) of action are unclear (7). Here we identify a pertussis-like AB₅ protein (EcPltAB) from clinical E. coli isolates that is related to the typhoid and ArtAB toxins observed in typhoidal and nontyphoidal Salmonella serovars. We provide structures that confirm that pertussis-like toxins are secreted as inactive forms in which an intramolecular disulfide holds an occluding C-terminal tail within the NAD⁺-binding site. This conserved disulfide also serves as a redox switch that senses host cell entry, with reduction of the bond allowing displacement of the occluding C-terminal residues, facilitating NAD⁺ binding and maturation of catalytic activity. At a global level, the existence of EcPltAB-type proteins, and their relatives (55) expands the family of known bacterial pertussis-like toxins capable of modulating the human immune system. Furthermore, we show that although AB₅ proteins are segregated into evolutionarily related enzyme families, these may not always modify the same residues within host proteins.

Results

Identification of EcPltAB

The frequency with which pathogenic bacterial strains are isolated from clinical settings does not necessarily correlate with their representation in genomic databases, yet it remains a useful proxy measure. We thus determined the prevalence of genes encoding AB₅-type toxins among complete and draft E. coli genome sequences available on the NCBI database. E. coli genomes were queried using previously identified A and B subunit sequences. As expected, most genes discovered corresponded to previously known E. coli toxins; by far the most prevalent were the Shiga toxins produced by Shiga toxigenic E. coli, proteins whose action can cause hemolytic-uremic syndrome following infection, identified in 19% of genomes (supplemental Fig. S1). The next most prevalent were toxins from the cholera family LT-I and LT-II heat-labile enterotoxins secreted by enterotoxigenic E. coli (Fig. 1), identified in ~8% of the genomes. The properties of these enteric E. coli toxins are well-established (4, 5, 11). However, the third most common set of AB₅ genes identified was a series of related pertussis-like proteins found in ExPEC genomes, particularly from phylogroup B2 (Fig. 1). The B subunit of these toxins shared 69% sequence identity to ArtB and the upstream A subunit 70% identity to ArtA, two components of a pertussis-like toxin produced by Salmonella typhimurium DT104 (16). In contrast to these Salmonella and Escherichia orthologs, the archetype pertussis toxin has an expanded set of four glycan-binding B subunits (named S2–S5) associated with a single catalytic A or S1 subunit. The Salmonella ArtAB protein is an active ADP-ribosyltransferase (17), whereas a related typhoid toxin additionally serves as a cargo carrier for an accessory cytotoxic distending toxin (7). Our analysis indicates that the E. coli Plt toxins are more evolutionarily diverse than the currently identified Salmonella variants, with at least three semi-separated lineages sharing between 70 and 80% sequence identity. We refer to the product of these pertussis-like genes as E. coli-pertussis-like toxins (EcPlt).
and an absence of growth (see supplemental Fig. S2, although those exposed to clustered growth (19). Over an 18-h period, control-treated lowly allowed by an accumulative interruption of signaling resulting in the characteristic response profile of pertussis toxin involves a slow cellular entry mechanism followed by an interdependent interruption of signaling resulting in clustered growth (19). Over an 18-h period, control-treated cells remained unaffected and continued to proliferate, although those exposed to EcPltAB displayed cell clustering and an absence of growth (see supplemental Fig. S2, a–c) compared with buffer-alone controls (supplemental Fig. S2d). Next, we performed a glycan array analysis to determine the specificity of EcPltAB (see supplemental Table S1). Its B subunit displayed avidity for a broad range of branched eukaryotic glycans, especially those with N-acetyllactosaminic acid (Neu5Ac) termini, thus implying EcPlt targets a similar range of cell types as typhoid toxin (7).

Next, we sought to establish which eukaryotic proteins were targeted by EcPltA. The EcPltA toxin shares 50–70% identity with Salmonella ArtA proteins and 31% identity with the pertussis toxin’s catalytic S1 subunit, two known ADP-ribosyltransferases that modify the α subunits of inhibitory trimeric GTP-binding proteins (17, 20). We set up an assay to identify equivalent EcPltA substrates by using biotin-conjugated NAD⁺ as substrate for the toxins. In such assays, ADP-ribosylation also adds a biotin tag to protein substrates that can then be imaged by Western blotting using streptavidin-horseradish peroxidase. Holotoxin (EcPltAB or PT) versions of this assay are dependent upon toxin activation and ADP-ribosylation rate, although when the assay is performed with constitutively active proteins (EcPltA or PT S1), only the ADP-ribosylation rate is measured.
Using radiolabeled substrate, other groups (20) have shown that the PT holotoxin is activated by the presence of ATP and reducing agents. Our own ADP-ribosylation assay recapitulates this result with a 41-kDa band being modified by PT in crudely purified trimeric G-protein brain extracts or Vero cell lysates under identical conditions (Fig. 2). A similar band was observed in reactions containing the EcPltAB holotoxin (Fig. 2). In both reactions, a major band at ~41 kDa was ADP-ribosylated and migrated close to the molecular weight of PT-treated Gi proteins (Fig. 2b, left panel). To confirm that EcPtA targeted Gi subunits, we cloned two HsGa_i3 constructs (the most readily expressed isoform): the full-length protein and a more soluble construct lacking the N-terminal aliphatic helix (HsGa_i3ΔN). Following expression and purification, full-length HsGa_i3 acted as substrate for the toxin’s ADP-ribosyltransferase activity and migrated at a similar molecular weight to a band modified in Vero cell extracts, whereas other proteins within the Coomassie-stained lysate did not (Fig. 2b, right panel). Both the full-length protein and Ga_i3ΔN-mutant served equally well as substrates for PT or EcPlt (supplemental Fig. S3), the latter Ga_i3ΔN construct was favored for routine assays due to its enhanced stability.

PT is responsible for many of the pathogenic features of B. pertussis. However, other Bordetella species also contain...
ptx-like genes yet fail to produce the toxin because of inactive promoters (21). Using our assay, we sought to identify whether ADP-ribosyltransferase activity was detectable in the supernatant of UPEC strain PA26B. UPEC PA26B was cultured in the presence of prophage-inducing agents. After 12 h of growth, bacterial cells were removed by filtration, and the supernatant was tested for ADP-ribosyltransferase activity using HsGα3N as a substrate. In control reactions employing purified EcPltAB, a robust ADP-ribosyltransferase response was observed using 20 nM pure toxin (Fig. 2c, 1st lane). Prior to commencing the reaction, the supernatant of PA26B cells was seen to contain a natively biotinylated 20-kDa protein, but this was readily distinguishable from our host HsGα3N target (Fig. 2c, 2nd lane). When HsGα3N was added to the filtered supernatant of PA26B, we observed ADP-ribosylation of the substrate (Fig. 2c, 3rd lane) suggesting, but not conclusively proving, that EcPltAB is actively expressed. The B subunit of the typhoid toxin impacts cell-signaling pathways independent of its associated catalytic A subunits (22). To determine whether the growth-arrest phenotype induced by EcPltAB resulted from its enzymatic activity, inactivating mutations were incorporated into the A subunit. A highly conserved (Q/E)XE motif is present in many ADP-ribosyltransferases and is essential for activity (23) (residues 116QNE118 in EcPltA). Mutation of residue Glu-118 to an aspartic acid residue resulted in a requirement for 1000-fold higher concentrations of toxin to induce the same level of growth arrest as wild-type EcPltAB toxin (Fig. 2d). Furthermore, a Q116D,E118D double mutant that retains a wild-type B subunit showed no effect on growth and was indistinguishable from control reactions even at 50 µg/ml toxin (Fig. 2d and supplemental Fig. S2e), and as discussed later, this mutant displayed no discernible enzymatic activity (Fig. 7c). EcPltAB thus appears to be expressed by ExPEC, and its ADP-ribosyltransferase activity is cytotoxic to mammalian cells.

The presence of ATP, phosphate, and detergent assists with activation of the PT S1 subunit by promoting dissociation from its pseudopentamer and increasing its exposure to the local microenvironment (24). ATP binds within the central pseudopentameric pore of the pertussis toxin, destabilizing the quaternary structure (25). We sought to determine whether the activation requirements for the EcPltAB toxin were similar. Our assay recapitulated the finding that reducing agents activate PT and that this process is synergistically enhanced by the presence of ATP (Fig. 3a, 1st to 5th lanes). Like PT, the EcPltAB holotoxin is inactive when oxidized, and higher concentrations of reducing agent result in increased catalytic activity in vitro (Fig. 3a, 6th to 8th lanes). However, ATP did not synergistically enhance this process (in Fig. 3a, 4th lane is 95% more intense than 2nd lane, although the intensities of the 7th and 9th lanes are within 1%). The ATP-binding site of the PT pseudopentamer appears absent within the homopentameric EcPltAB. We examined whether the nature of the reducing agent affected EcPltAB activation by repeating the ADP-ribosylation assays in the presence of intracellular (2 mM) or serum (2 µM) concentrations of glutathione using different redox ratios. Reduced glutathione alone was ~20% more effective at inducing toxin activation.
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in vitro than an equivalent concentration of TCEP (Fig. 3c), yet even small quantities of oxidized glutathione (GSSG) drastically slowed activation. Thus, although some characteristics are shared, the sequence divergence that exists between pertussis toxin and pertussis-like toxins results in differences in their respective activation mechanisms.

Protein substrates

PT targets the α subunits of human inhibitory trimeric G-proteins by transferring the ADP-ribose moiety of NAD⁺ onto a conserved cysteine four residues from their C termini. PT-sensitive substrates primarily belong to the heterotrimeric G/o protein subfamily, which in humans includes the 40.5-kDa proteins Gαi1, Gαi2, Gαi3, and Gαo, as well as the equivalent subunit from transducin. The Salmonella ArtA pertussis-like toxin modifies this same set of proteins (17), indicating some degree of target conservation. As the HsGαi3 was shown to be an EcPltA substrate, we sought to test for activity against the remaining trimeric Gαi proteins enriched from brain lysate using equal amounts of a commercial Gαi/o substrate for either toxin. When EcPltA targeted another cysteine residue, we could completely abrogate the INSL5-stimulated ERK1/2 subunits are recruited to RXFP4. We first confirmed that PT inhibition is disrupted by EcPltAB had on GPCR-signaling pathways in cells. The Relaxin family peptide 4 receptor (RXFP4) is a GPCR that recruits Gαi/o subunits upon binding its ligand: insulin-like peptide 5 (INSL5); the induced nucleotide exchange inhibits forskolin-stimulated cAMP production and promotes ERK phosphorylation (29), a response that can be abolished through PT pretreatment. Gαi/o proteins with a C351I mutation (mGαi3) are PT-insensitive and can be used to rescue the PT-abolished GPCR-signal transduction. PT could still act on methylated HsGαi3 substrate but at a slightly reduced rate compared with controls (Fig. 4c).

To identify the residue(s) within HsGαi3 modified by EcPltA, the reaction product was subjected to MS–MS analysis. ADP-ribosylation results in a +541-Da mass shift to precursor peptides, and fragmentation of the pyrophosphate bond also liberates a characteristic 348.1-Da adenine monophosphate during product ion scans (supplemental Fig. S4). We obtained full peptide coverage of HsGαi3 and could not detect any evidence of cysteine modification, instead ADP-ribosylated modification sites were identified in HsGαi3 C-terminal sequences: 331NVQVFVDATVDVII-ADP-ribosylated--NNLK349 and 346NN-ADP-ribosylated--LKECGLY354. Thus, EcPltA ADP-ribosylates two residues (Lys-345 and Asn-347) at the C terminus of HsGαi3, sites distinct from PT modification of Cys-351. We next constructed three mutants, HsGαi3(K345A), HsGαi3(N346A), and HsGαi3(N347A), and tested their suitability as substrates for both toxins. HsGαi3(K345A) was seen to be resistant to ADP-ribosylation by EcPltA, yet it was readily modified by the constitutively active PT S1 (Fig. 4d); HsGαi3(N346A) was readily ADP-ribosylated by either toxin and HsGαi3(N347A) by neither toxin (Fig. 4e). It is noteworthy that mutation of either Lys-345 or Asn-347 to alanine also reduced enzymatic activity against the other site, suggesting a concerted mechanism of recognition. Together, these experiments suggest that different pertussis-like toxins can target distinct but nearby residues within the C termini of Gαi proteins.

Gαi/o recruitment to GPCRs is disrupted by EcPltAB

We next examined what effect EcPltAB had on GPCR-signaling

Pathways in cells. The Relaxin family peptide 4 receptor (RXFP4) is a GPCR that recruits Gαi/o subunits upon binding its ligand: insulin-like peptide 5 (INSL5); the induced nucleotide exchange inhibits forskolin-stimulated cAMP production and promotes ERK phosphorylation (29), a response that can be abolished through PT pretreatment. Gαi/o proteins with a C351I mutation (mGαi3) are PT-insensitive and can be used to rescue the PT-abolished INSL5-stimulated ERK1/2 response to determine which Gαi/o subunits are recruited to RXFP4. We first confirmed that PT could completely abrogate the INSL5-stimulated ERK1/2 response within Chinese hamster ovary (CHO)-RXFP4 cells transfected with the pcDNA3 vector control (Fig. 5a); an identical result was obtained with EcPltAB pretreatment (Fig. 5a). Next, we demonstrated that ERK1/2 responses were partially restored in PT-treated cells transfected with mGαi3, mGαi, mGαi,i1, mGαi,i2, or mGαo which are PT-insensitive and can be used to rescue the PT-abolished INSL5-stimulated ERK1/2 response within Chinese hamster ovary (CHO)-RXFP4 cells transfected with the pcDNA3 vector control (Fig. 5a); an identical result was obtained with EcPltAB pretreatment (Fig. 5a). Next, we demonstrated that ERK1/2 responses were partially restored in PT-treated cells transfected with mGαi3, mGαi, mGαi,i1, mGαi,i2, or mGαo which are PT-insensitive and can be used to rescue the PT-abolished INSL5-stimulated ERK1/2 response within Chinese hamster ovary (CHO)-RXFP4 cells transfected with the pcDNA3 vector control (Fig. 5a); an identical result was obtained with EcPltAB pretreatment (Fig. 5a). Next, we demonstrated that ERK1/2 responses were partially restored in PT-treated cells transfected with mGαi3, mGαi, mGαi,i1, mGαi,i2, or mGαo which are PT-insensitive and can be used to rescue the PT-abolished INSL5-stimulated ERK1/2 response within Chinese hamster ovary (CHO)-RXFP4 cells transfected with the pcDNA3 vector control (Fig. 5a); an identical result was obtained with EcPltAB pretreatment (Fig. 5a). Next, we demonstrated that ERK1/2 responses were partially restored in PT-treated cells transfected with mGαi3, mGαi, mGαi,i1, mGαi,i2, or mGαo which are PT-insensitive and can be used to restore INSL5-stimulated ERK1/2 responses in cells treated with EcPltAB (Fig. 5, b–f), underlining that within cells the residue(s) on Gαi/o targeted by EcPltAB differ from the canonical Cys-351 ADP-ribosylated by PT and that once modified the GPCR signaling is disrupted.
As Go1α subunits carrying the PT-resistant C351I mutant remain sensitive to EcPltAB treatment, we next sought to identify alternative EcPlt-resistant mutations. To do so we monitored the cAMP concentration in cells responding to the adenylyl cyclase activator forskolin (supplemental Fig. S5a). This stimulatory response can be counteracted by inhibitory trimeric G-proteins coupled to INSL5-induced RXFP4 signaling, which acts to restore cAMP levels to a near basal state (supplemental Fig. S5a). The RXFP4-transduced inhibitory signal can be abrogated by either toxin (supplemental Fig. S5a), and reconstituting C351I Go1α subunits allows partial recovery of the inhibitory signal in the presence of PT (supplemental Fig. S5, b–f) but not EcPltAB. In an attempt to achieve an equivalent response for the enterobacterial toxin, we mutated its two identified ADP-ribosylation sites within Go1αN to alanine (N347A and K345A) and repeated the experiment (supplemental Fig. S5, g and h). Neither HsGo1α(N347A) nor HsGo1α(K345A) facilitated the recovery of PT-treated cells. In contrast, EcPltAB treatment of cells transfected with HsGo1α(N347A) displayed a 26 ± 5% difference in the cAMP response in the presence of

![Figure 4. Proposed model of the pertussis-like toxins transfer target.](image-url)
INSL5/forskolin or forskolin alone (supplemental Fig. S5g), consistent with the N347A mutant providing a degree of protection for EcPltAB but not for PT. In the same experiment, HsGαi3(K345A) did not obviously allow recovery of INSL5-mediated signaling (Fig. 7h).

Structure of the secreted EcPltAB holotoxin

To understand the secreted ExPEC toxins in more detail, we determined the 2.4 Å crystal structure of the EcPltAB holotoxin (see supplemental Methods and Table 1 for details). This heterohexameric complex resembles a blunted pyramid in which the EcPltA subunit sits atop a pentameric B subunit base, thereby adopting an overall quaternary structure of the AB5 family (Fig. 6a). In the secreted oxidized state, the enzymatic A subunit contains a single intermolecular disulfide bond between the two conserved cysteines (Cys-41 and Cys-192, Fig. 6b); each of the five EcPltB subunits contains a further two intermolecular disulfides. EcPltA shares 52% sequence identity to the typhoid toxin’s A subunit (30) and 31% identity to the S1 subunit of pertussis toxin (9), its two closest known structural homologs (root mean squared deviation of 1.1 Å over 207 of 224 Cα’s; and 2.0 Å over 186 Cα’s, respectively PDBeFOLD). In all three holotoxins, the respective orientation of the A subunit atop the B pentamer is similar, with the Cα backbone structure of the EcPlt holotoxin and the lower AB5 section of the typhoid toxin overlaying over most of their length. The conserved intramolecular disulfide of pertussis group toxins holds their inhibitory C-terminal residues inside their nucleotide-bind-

Table 1

| Data collection and refinement statistics |
|------------------------------------------|
|                                       |
| Data collection                        | EcPltAB | EcPltA1 |
| Redox state                            | Oxidized | Reduced |
| Space group                            | P65     | P1     |
| Cell dimensions                        |          |
| a, b, c (Å)                            | 160.9, 160.9, 62.6 | 44.5, 55.7, 73.1 |
| a, b, γ (°)                            | 90.0, 90.0, 120.0 | 90.1, 104.1, 96.2 |
| Resolution (Å)                         | 4.1 (5.63) | 6.6 (30) |
| Rwp (%)                                | 12.1 (2.1) | 7.7 (2.6) |
| Completeness (%)                       | 99.9 (99.7) | 88.5 (87.7) |
| Total no. of observations              | 326,219 (45274) | 91,849 (12898) |
| No. of unique observations            | 38,825 (5619) | 55,479 (8027) |
| Multiplicity                          | 8.4 (8.1) | 1.7 (1.6) |

| Refinement statistics |
|-----------------------|
| Rwork (%)             | 19.2 (30.0) | 18.1 (26.1) |
| Rfree (%)             | 25.5 (34.3) | 22.7 (31.6) |
| No. of atoms          | 6380 | 5591 |
| Protein               | 1 (PO4) | 176 (4 NAD+, 2Na+) |
| Water                 | 64 | 598 |
| Ramachandran plot (%) | 94.0 | 97.5 |
| Most favored          | 94.0 | 2.5 |
| Allowed region        | 0.0 | 0.0 |
| Outlier               | 0.0 | 0.0 |
| B-factors (Å)         | 68.1 | 16.2 |
| Protein               | 0.01 | 0.01 |
| Root mean square deviation bonds (Å) | 1.37 | 1.18 |

* Rwork = Σ|Fcal| - |Fc||/Σ|Fcal|, * Rfree = Σ|Fobs| - |Fc||/Σ|Fobs| for all data except as indicated in Footnote c.

* 5% of data was used for the Rfree calculation.
ing sites (Fig. 6b), preventing catalytic activity prior to entry into the reducing environment of the host cytoplasm.

**Structure of the cytoplasmic NAD⁺-bound EcPltA**

To determine what structural changes occur following release of the C-terminal redox switch, we determined a 1.8-Å resolution structure of the constitutively active catalytic EcPltA subunit (residues 5–181) purified under reducing conditions (Table 1). As ART enzymes display off-target NAD⁺-glycohydrolase activity in the absence of substrate, we utilized the catalytically inactive mutant (Q116D,E118D) to slow this reaction and permit stable co-complex formation with the NAD⁺ substrate (30). These crystals diffracted in space group P1 with four complexes of NAD⁺-EcPltAcat within the asymmetric unit allowing independent models of the ART domain to be compared. The enzymatic core of the ART fold consists of ~150 amino acids and has a U-shape with a concave cleft creating the NAD⁺-binding site (Fig. 6c). The two β-sheets of EcPltA are on either side of this nucleotide-binding cleft: on one side is the β-sheet with strands s1, s3, s6, and s7 sandwiched between helix 3 and helix 1, and on the other side is the fold’s smaller s2, s5, and s4 β-sheet along with its h2, h4, and h5 helices. Unbiased omit maps showed NAD⁺ to be bound inside the U-shaped ART domain with two distinct binding modes observed, one extended and the other semi-extended (Fig. 7) (31). The extended conformation (supplemental Fig. S6a) dominates in the A, B, and C protomers and the semi-extended conformation in D (supplemental Fig. S6b). Both bound forms of NAD⁺ adopt highly folded nicotinamide mononucleotide (NMN) sections, whereas the adenine base transits between alternative aromatic stacking interactions. The common factor within the two distinct nucleotide conformations is the folded NMN group, and forcing the nucleotide to adopt this form is proposed to be an integral part of catalysis (32). The residues stabilizing the compact NMN are contributed from both sides of the binding cleft. Strand s1 provides a conserved phosphate-binding nucleo-
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phic amino acid (Arg-9) also found in PT, although the amide group of the nicotinamide moiety forms hydrogen bonds to the peptide backbone (Fig. 7a). On the other side of the cleft, residues within a semi-conserved “STS” motif within the s2 strand position the scissile bond for catalysis by clasping the nicotinic ring of NAD\(^+\) (actual residues 52ATT54, supplemental Fig. 56c). The inactivating E118D mutation of the catalytically essential glutamate shifts the negatively charged carboxyl away from the nucleotide (Fig. 7, a and b); if the Glu-118 rotamer observed in the wild-type holotoxin was to be maintained following reduction, its carboxyl group would be positioned within hydrogen-bonding distance of the N-ribose's 2'-hydroxyl (Fig. 7e). This interaction is highly conserved within ART enzymes and is thought to promote nicotinamide scission by stabilizing the formation of an oxocarbenium ion intermediate (33). The movement of the carboxyl group caused by the E118D mutation helps explain why this otherwise relatively conservative substitution is inactivating in the ART enzymes (Fig. 7c).

In the extended NAD\(^+\) conformation, the adenine base interleaves between the guanidinium group of Arg-13 and the indole ring of His-26. A similar interleaved interaction is seen in the NAD\(^+\)-bound cholera toxin structure (15). In contrast, the semi-extended NAD\(^+\) conformation has the adenine base stacked atop Tyr-67 (Fig. 7b). The Y67A mutation was seen to abrogate catalytic activity in the ADP-ribosyltransferase assays using HsGo (3), as substrate (Fig. 7c), but ~25% wild-type activity is retained by Y67F mutants suggesting it is the aromatic base-stacking role that is its more vital feature (Fig. 7c). Moreover, an S70W mutation, which would disrupt the adenosine-binding site of the semi-extended but not the extended conformation, also displayed minimal ADP-ribosyltransferase activity in vitro (Fig. 7, b and c) suggesting this conformation may play a role during catalysis.

Several EcPltA residues located at the intersubunit interface of the holotoxin become solvent-exposed following subunit dissociation; this includes Ile-111 on h5 and Ile-58 and Ser-56 on h3 (gray labels in Fig. 7b). To determine whether any such residues are also involved in protein interactions with the transfer target, they were mutated to large bulky residues to disrupt potential recognition interfaces. All mutations on the exterior face of h3 had no effect (Fig. 7c), whereas equivalent mutations on h5 (I111Y) were inactivating. This h5 helix is N-terminal to the catalytic (E/Q)XE motif, a region of the ART domain that in cholera and C3-like toxins corresponds to a conserved flexible loop between strands s4 and s5 known to be necessary for substrate recognition (34).

Redox-induced structural changes

We compared the inactive oxidized and active NAD\(^+\)-bound states of EcPltA (Fig. 7e; supplemental movie). This shows that the activation mechanism of pertussis-like toxins is fundamentally different from the loop-refolding mechanism of cholera toxin (15). The conserved intramolecular disulfide within the EcPltA-oxidized state (Cys-41–Cys-192) is located inside the NAD\(^+\)-binding cleft directly above the catalytic glutamate (Glu-118), Cys-192 being the C-terminal residue of the short inhibitory A2 helix. In the holotoxin, the binding pocket for the nicotinamide ring is filled by Phe-193 from h2A (Fig. 7d); subsequent A2 backbone residues then rise to thread through the cleft with Arg-199 and Arg-200 occupying the same site as the adenosine ring in the extended conformation (pink loop in Fig. 7d). The A2 loop subsequently doubles back on itself before entering the pentamer pore; during this descent, it blocks the adenosine-binding site near helix 3 for the semi-extended conformation (Fig. 7b). The nucleotide-binding site of EcPltA is made from dynamic regions of the protein that move following release of the inhibitory C-terminal residues as well as regions that remain stationary. A static floor capable of accommodating either NAD\(^+\) or the A2 peptide includes the adenine base-interleaving residues Arg-13 and His-26 (Fig. 7a), the phosphate-binding nucleophile Arg-9, and the nicotinamide “STS” clasp equivalent residues. In contrast, structural movements are required to form the side walls of the NAD\(^+\)-binding site. This is necessary as the occluding C-terminal helix is more bulky than the substrate nucleotide.

After the redox-sensing disulfide is reduced, Cys-41 and the remainder of the active-site loop undergo a ~2.5-Å rigid-body inward movement toward the adenosine ribose and phosphate (Fig. 7e). This loop movement carries the h2 helix with it until it abuts the 3’-hydroxyl of the N-ribose, and in doing so the catalytic residue His-35 (35) is shifted toward the scissile N-glycosidic bond (Fig. 7, a and e) where it may interact with Glu-118. Following NAD\(^+\) binding, helix 3 pivots about its N terminus resulting in a maximal displacement from the oxidized form of ~8.2 Å (Fig. 7e). This movement allows the side chains of Tyr-67 and Ile-63 to move in and neighbor the nicotinamide ring. These NAD\(^+\)-binding associated movements are likely to be conserved features of activation within typhoid toxin and pertussis toxin.

Discussion

We provide the structural details of the E. coli pertussis-like toxin in its intracellular reduced form, detailing different substrate-binding modes and the redox-induced structural changes that occur during activation. In the oxidized state, the disulfide-linked C terminus of the A subunit inactivates the protein by occluding the NAD\(^+\)-binding site (Fig. 6), a mechanism clearly conserved within pertussis toxin as suggested by modeling studies (36). Loss of the inhibitory A2 domain from within the nucleotide-binding cleft allows significant lobe closure and reorganization of residues about NAD\(^+\) particularly within helices h2, h3, and the active-site loop. This contrasts with the activation mechanism of the cholera toxin whose A2 domain is largely external to the catalytic cleft and requires no
lobe movements upon activation, but it involves significant loop restructuring (30). Our high-resolution co-crystal structure details NAD⁺ binding in two distinct conformations: the dominant extended conformation is similar to that observed in nucleotide-soaked CT crystals (30), and a second lower-occupancy semi-extended conformation also exists and may play a role during catalysis as C-terminal h₃ mutations predicted to disrupt the base-stacked adenine abrogate activity.

The residues within h₂ are relatively well-conserved, suggesting the redox-induced movement of this structural element and the restructuring of the preceding active-site loop are a conserved features of pertussis-like proteins. In contrast, helices h₃ and h₅ show more significant structural divergence between the pertussis and the enterobacterial holotoxins, with these regions more likely involved in binding to the transfer target. GPCR binding is disrupted by pertussis toxin through modification of the Gαᵣ residue Cys-351, and the toxin is highly specific and unable to modify mutants lacking this residue (Fig. 6). The extent to which pertussis-like toxins is conserved could have correlated with conservation of target chemistry, yet we show EcPlt does not modify cysteine residues within Gαᵣ proteins but instead a lysine and asparagine several residues away. Moreover, our cAMP experiments confirmed that EcPltAB enters cells and inhibits the action of Gαᵣ proteins via a residue other than Cys-351 (Fig. 4). This is not unprecedented; human poly(ADP-ribose) polymerase 1 initiates polymer formation on C-terminal chromatin lysines (37), but ADP-ribosylation at such sites has not previously been described within bacterial toxins nor in human Gαᵣ subunits. Why would this site be targeted? GPCR-Gαᵣ-protein coupling occurs when the predominantly helical C terminus of the Gαᵣ subunit inserts into a pocket built from the cytoplasmic facing ends of the receptor TM helices. The co-complex structure of opsins in complex with the C terminus of transducin (38) shows that Cys-351 penetrates deep into the binding pocket, highlighting why pertussis toxin’s modification would be incompatible with receptor coupling. As the C terminus of receptor-bound Gᵣ subunits are predominantly helical, Asn-347 lies a turn beneath Cys-351 interacting with the same transmembrane helix (TM3). Lys-345 is on the opposing side of the G-protein’s C terminus interface with TM6. Thus, both Asn-347 and Lys-345 reside closer to the cytoplasmic “mouth” of the receptor’s binding pocket than Cys-351 when coupled to their cognate GPCRs.

EcPltA-catalyzed ADP-ribosylation may thus block receptor recruitment of inhibitory heterotrimeric G-proteins using a related but distinct mechanism to that used by pertussis toxin. Our attempts to create an EcPlt-resistant Gαᵣ subunit were partially successful (supplemental Fig. S5) and could be interpreted as indicating that the Asn-347 modification may dominate over the Lys-345 site in vivo. In vitro, both Lys-345 and Asn-347 were required for EcPltA to modify HsGαᵣᵢ, so both residues may be involved in toxin:G-protein binding. Peptides consisting of the 10–20 C-terminal residues of HsGαᵣᵢ can act as minimal pertussis toxin substrate inhibitors (39) suggesting the entire protein is not necessarily required for recognition. Our work might imply that EcPlt has shifted its respective recognition motif within Gαᵣ proteins several residues downstream from that of PT with Asn-347 playing distinct roles in both EcPlt and PT toxins recognition interfaces; we note that Asn-347 appears to be more essential for the action of EcPlt as its mutation did not provide sufficient protection to reconstitute signaling in the presence of PT. Further work will be required to validate our reconstitution experiments; mutants that disrupt the toxin–G-protein interface may also influence GPCR binding and/or GTPase activity.

Finally, pertussis toxin has become a valuable tool for dissecting GPCR pathways, and the identification of an expanded toolkit of pertussis-like proteins with varied inhibitory mechanisms may permit additional experiments to be performed. Given the proven utility of PT in this role, it may be beneficial to further characterize the behavior of other pertussis-like toxins in reconstituted systems to fully understand how this toolset functions.

**Experimental procedures**

**Materials**

All chemicals were purchased from Sigma unless otherwise stated. Mouse INSL5 was synthesized and purified at The Florey Institute of Neuroscience and Mental Health (Melbourne, Australia) by Dr. Akhter Hossain (40). FBS, DMEM Ham’s/F12, Lipofectamine 2000, and PT were all from Life Technologies, Inc. AlphaScreen SureFire® ERK1/2 phosphorylation kits, acceptor beads, and donor beads were all from PerkinElmer Life Sciences.

**Bioinformatic analysis and bacterial strains**

*E. coli* strains containing AB₃-type toxin-like genes were identified from an *E. coli* dataset (EcoDS) comprising 1700 genome sequenced strains deposited in the NCBI database up to January 2014 (56), using a querying dataset based on characterized AB₃ toxins (Art (16), Shiga (41), subtilase (42), and cholera (43) toxins). Sequence comparisons were performed using the FASTA36 package (44); ORFs with an amino acid sequence identity >75% of the toxin subunits were extracted from the public dataset. A neighbor-joining tree was generated using the concatenated AB toxin subunits with ClustalW (45). The phylogenetic group was determined using an *in silico* tripod scheme (46, 47), and MLST analysis was performed according to a seven-gene scheme described previously (48). UPEC strain PA26B was isolated from a patient presenting with a urinary tract infection at the Princess Alexandra Hospital (Brisbane, Australia) (54).

**Toxin expression**

Toxin expression was induced by stressing PA26B cells and a control K12 laboratory strain, and overnight cultures grown at 37 °C were diluted 1:40 into 40 ml of LB broth without antibiotics and grown for 4 h at 37 °C before addition of mitomycin C or 10 mM H₂O₂. After 24 h of growth, all proteins secreted into the media were harvested and clarified by passing through a 0.2-μm filter. 10 nm bovine catalase was added to the supernatant of cells stressed by H₂O₂.

**Cytotoxicity assay**

Kidney epithelial cells from the African green monkey (Vero cells) or human embryonic kidney (HEK293T) were expanded...
Cloning

A co-expression vector for the EcPltAB toxin was created as in Ng et al. (12). Briefly, both the full-length EcPltA and EcPltB genes were chemically synthesized (GenScript), with the addition of restriction sites Ndel and Xmal and Xbal and Xhol flanking the EcPltA and EcPltB open reading frames, respectively. The EcPltA gene containing its native signal sequence was cloned directly into pBAD18. EcPltB was subcloned into pET-23 (Novagen) via Xbal and Xhol sites, resulting in a protein with a C-terminal poly-His tag and N-terminal PelB signal sequence; this construct was used for the expression of unliganded EcPltB pentamer. To produce the co-expression vector for EcPltAB, the ribosome-binding site and EcPltB was excised from pET-23 using the Xbal and HindIII restriction sites prior to being cloned downstream of EcPltA within the pBAD vector.

The catalytic domain of the pertussis toxins S1 unit, EcPltA, and human HsGαi3 proteins were cloned into a pET28 expression vector fused to a hexahistidine tag through a human rhinovirus 3C cleavage site. The constitutively active EcPltA construct encompassed residues 5–181. The HsGαi3 constructs encompassed residues 034–354 or 005–354. Mutants used in enzymatic assays were created by oligonucleotide-directed mutagenesis followed by DpnI digestion of the parental vector, with all resulting constructs confirmed by sequencing.

Protein expression and purification

For protein expression, EcPltAB was transformed into E. coli BL21(DE3) cells and then grown in LB at 37 °C containing 100 μg/ml ampicillin to A600 = 0.6 followed by a 4-h induction at 37 °C with 0.2% L-arabinose. Cell culture pellet was collected with all resulting constructs confirmed by sequencing.

Cytotoxicity assays were performed by adding recombinant EcPltAB holotoxins at 50 μg/ml to 500 pg/ml. Cells were left for 18 h and then stained with crystal violet and imaged. The surface area covered by the cells was determined by the number of blue pixels within each image normalized against buffer controls.

Enzymatic assay

Activity assays were performed using 6-Biotin-17-NAD+ (BPS Biosciences 80610) as a substrate for the modification of different G-protein substrates. The resulting biotin adducts were visualized using streptactin-conjugated horseradish peroxidase (Bio-Rad 161-0381). Briefly each reaction mix contained ~1 μM protein substrate, which consisted of recombinant HsGαi3 purified trimeric bovine brain G-proteins (EMD Millipore 371739), or whole Vero cell lysate. Standard assay conditions consisted of ~1 μM G-protein in 20 mM HEPES, pH 7.0, 140 mM NaCl, 5 mM TCEP, and 10 μM biotin-NAD+, which was incubated for 5 min at 37 °C with 20 μM toxin. Holotoxin assays were performed with up to 100 mM TCEP to induce activation and consisted of PT purified from Bordetella lysate (Sigma P7208) or recombinant EcPltAB. Reactions were stopped by adding SDS-loading buffer and heating at 100 °C for 5 min before analysis by SDS-PAGE. Analysis by 2D gel electrophoresis was done by the Proteomics Facility, Monash University; samples were separated in the 1st dimension over broad pH gradient 3–10 before separation in the 2nd dimension by SDS-PAGE (gradient of 9–16% acrylamide). 1D and 2D gels were processed for analysis by Western blot; proteins were transferred to a membrane prior to being visualized using streptactin-HRP and Pierce chemiluminescence substrate. Assays performed with recombinantly produced G-proteins were subsequently stained by Coomassie to ensure equal loading. All assays were repeated three times to ensure reproducibility. Densitometry was performed using an Epson V700 scanner and data quantification via ImageQuant.

Activation mechanism of pertussis-like toxins

To 40–50% confluency in 10-cm² dishes covered with 2 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. Cytotoxicity assays were performed by adding recombinant EcPltAB holotoxins at 50 μg/ml to 500 pg/ml. Cells were left for 18 h and then stained with crystal violet and imaged. The surface area covered by the cells was determined by the number of blue pixels within each image normalized against buffer controls.

In HBS with 20 mM imidazole and 1 mM TCEP, then lysed by adding 1 mg/ml lysozyme and DNase, and then sonicated. Cleared lysate was applied to a Ni-NTA affinity column and washed extensively before eluting in 20 mM HEPES, pH 7.0, 250 mM NaCl, 400 mM imidazole, 1 mM TCEP. The protein’s hexahistidine tag was cleaved by incubating the eluted protein with 0.1 mg/ml GST-fused HRV 3C overnight at 4 °C. The HRV 3C protease was removed by passing over 0.1 ml of GSH-agarose resin with the flow-through subsequently purified by gel filtration on a Superdex S75 16/60 (GE Healthcare). The constructs eluted as a single peak corresponding to monomeric EcPltA. The pertussis toxin’s catalytic S1 domain was purified using a similar manner.

Expression of the different HsGαi3 constructs was identical to that of EcPltA except they required 0.01% (v/v) Triton X-100 during HRV 3C cleavage, and the protein was buffer-exchanged into 20 mM HEPES, 50 mM NaCl, 1 mM TCEP to allow HiTrap Q purification prior to gel filtration. When HsGαi3 mutants were being purified for enzymatic assays, chromatographic systems and columns were purged with two intervening dummy runs: one of 0.5 M NaOH followed by 1 M HEPES, pH 7.0. When required, HsGαi3ΔN was methylated using a reductive alkylation kit (Hampton Research HR2-434) or carbamidomethylated with iodoacetamide (Pierce 90034) according to the manufacturer’s instructions prior to the ion-exchange column during purification.

For protein expression, EcPltAB was transformed into E. coli BL21(DE3) cells and then grown in LB at 37 °C containing 100 μg/ml ampicillin to A600 = 0.6 followed by a 4-h induction at 37 °C with 0.2% L-arabinose. Cell culture pellet was collected and resuspended in HBS with 20 mM imidazole and 1 mM TCEP, then lysed by adding 1 mg/ml lysozyme and DNase, and then sonicated. Cleared lysate was applied to a Ni-NTA affinity column and washed extensively before eluting in 20 mM HEPES, pH 7.0, 250 mM NaCl, 400 mM imidazole, 1 mM TCEP. The protein’s hexahistidine tag was cleaved by incubating the eluted protein with 0.1 mg/ml GST-fused HRV 3C overnight at 4 °C. The HRV 3C protease was removed by passing over 0.1 ml of GSH-agarose resin with the flow-through subsequently purified by gel filtration on a Superdex S75 16/60 (GE Healthcare). The constructs eluted as a single peak corresponding to monomeric EcPltA. The pertussis toxin’s catalytic S1 domain was purified using a similar manner.

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Activation mechanism of pertussis-like toxins

Crystallization and data collection

The EcPltAB holotoxin crystals were obtained by setting up a 1:1 ratio hanging drop experiments with 16 mg/ml protein over a reservoir of 0.2 M MgCl₂, 0.1 M Tris-HCl, pH 8.5, 15% (v/v) polyethylene glycol 3350 at 293 K; crystals grow for 2–3 weeks and are then harvested by flash-frying in liquid nitrogen after soaking in a cryoprotectant solution containing an additional 25% (v/v) glycerol prior to data collection. EcPltAB crystals diffracted to 2.35 Å in space group P6₅ with one heteroadditive 25% (v/v) glycerol prior to data collection. Grown after soaking in a cryoprotectant solution containing an additional 25% (v/v) glycerol prior to data collection, the crystals were eventually grown as oddly shaped clusters that needed to be fragmented into single pieces prior to freezing in a reservoir complemented by a reservoir of 0.2M MgCl₂, 0.1M Tris-HCl, pH 8.5, and 15% (w/v) polyethylene glycol, 0.1M Li₂SO₄. Crystals clusters were broken into single pieces prior to freezing in a reservoir complemented by 4–7% (v/v) polyethylene glycol, 0.1M Li₂SO₄. Crystals clusters were broken into single pieces prior to freezing in a reservoir complemented with 25% (v/v) glycerol and flash-frozen in liquid nitrogen, subsequently diffracting in a monoclinic space group to 1.8 Å with four molecules in the asymmetric unit. A molecular replacement solution was obtained using a trimmed version of the holotoxin’s A1 subunit as a model with four molecules found within the asymmetric unit. In protomers A–D, clear electron density was observed for residues 4–174, and there were few differences in the Cₙ backbone structure among protomers except for a shift in protomer D within the loop C-terminal to h3. The final model contains four NAD⁺ molecules and all but the C-terminal residue of the construct.

Data completeness for the EcPltA in space group P1 was a little lower than ideal. When PT or EcEPlt dissociates from their B subunits, they mimic unfolded proteins to translocate toward the cytosol. In such structures h5 is highly flexible so crystals often grew as oddly shaped clusters that needed to be fragmented. In four of the molecules in the asymmetric unit, the h5 loop adopts different forms; moreover, such conformations varied when data were collected at different points in the crystal. This structural complexity caused issues for merging of datasets, and crystals showed some radiation damage after 150–200°, and the dataset was collected from multiple points on a single crystal.

Glycan array analysis

Purified EcPltAB protein at 1 or 100 µg/ml was applied to a PA_v51 glycan microarray at the Consortium of Functional Glycomics Protein—Glycan Interaction Core at Emory University, and binding was detected using Alexa488-conjugated anti-hexahistidine antibody. The data are reported as average relative fluorescence units of four of six replicates (after removal of the highest and lowest values) for each glycan represented on the array.

Mass spectrometry analysis

HsGαi₃ at 2 mg/ml was treated for 1 h with a 100:1 molar ratio of EcPltA in 20 mM HEPEs, 150 mM NaCl, 2.5 mM NAD⁺, 0.5 mM TCEP, pH 7.5. After the reaction, the protein was diluted into 20 mM HEPEs, 50 mM NaCl and then bound to a HiTrap Q column and eluted under a 20-mL gradient to 1 M NaCl to remove unreacted NAD⁺. The reaction product was diluted into 100 mM (NH₄)HCO₃ and heated to 95°C for 5 min; after cooling to room temperature, the sample was divided in two with fresh chloroacetamide added to one fraction at a final concentration of 5 mM for 30 min. Trypsin was then added at 1:50 ratio with the digestion allowed to proceed for 18 h at 37°C, and the digestion was finally quenched with formic acid at a final concentration of 0.25% (v/v). The resulting peptides were analyzed by LC-MS/MS using the QExactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled on line with an RSLC nano-HPLC (Ultimate 3000, Thermo Fisher Scientific) (52). MS/MS data analysis was done with MaxQuant software (build 1.5.2.8). MS data were analyzed using MaxQuant (53) searched against the human and E. coli K12 strain protein sequences taken from UniprotKB (March, 2015). ADP-ribosylation was defined as modification of a mass of 541.0611088 Da on with diagnostic peaks of 438.03699 (m8), 348.07036 (m6), 250.09346 (m3), and 136.06177 (m1) and the corresponding natural loss peaks. The search parameters include enzyme specificity of trypsin with three missed cleavages. Search parameters also allowed for cysteine carbamidemethylation, methionine oxidation, or ADP-ribosylation on cysteine, lysine, arginine, glutamate, aspartate, and asparagine. All other search parameters were set to the default settings.

Cell culture and transient transfection

CHO cells stably expressing RXFP4 (CHO-RXFP4) were a gift from Associate Professor Ross Bathgate (Florey Institute of Neuroscience and Mental Health, Melbourne, Australia). Cells were grown and maintained in 175-cm² flasks in DMEM/F-12 medium supplemented with 5% (v/v) fetal bovine serum (FBS) at 37°C in humidified air containing 5% CO₂. cDNA constructs for G-protein α subunits (Gα₁₆, Gα₁₂, Gα₁₃, or Gα₁₅) containing the C351I mutation were from the cDNA Resource Center (Bloomsberg, PA). HsGαi₃ containing K345A and N347A mutations were generated via site-directed mutagenesis using wild-type HsGαi₃ pcDNA3 vector as template. HsGαi₃ constructs carrying the mutations were transiently transfected into CHO-RXFP4 cells grown on 6-well plates using Lipofectamine 2000 as per the manufacturer’s protocol.

ERK1/2 phosphorylation

18 h after transfection, CHO-RXFP4 cells were trypsinized and seeded onto 96-well plates at a density of ~50,000 cells per well and incubated for 6–8 h in complete media to allow cell adhesion. Cells were then incubated with PT, EcPltAB, or control (serum-free DMEM/F-12) for 18 h before stimulation with mouse INSL5 (0.1 to 100 nM) for 5 min and then lysed. Phosphorylation of ERK1/2 (Thr-202/Tyr-204) was detected using
the AlphaScreen SureFire® kit. In brief, 4 µl of cell lysates was added to a white 384-well microplate (ProxiPlate; PerkinElmer Life Sciences) containing 5 µl of acceptor bead mix (40 parts reaction buffer/10 parts activation buffer/1 part protein A acceptor beads) and incubated for 2 h at 23 °C. Then, 2 µl of donor bead mix (20 parts dilution buffer/1 part streptavidin-coated donor beads) was added and incubated for 2 h at 23 °C. All additions and incubations were carried out under low light conditions to avoid photobleaching. Fluorescence readings were made using the Envision multilabel plate reader (PerkinElmer Life Sciences; excitation = 680 nm; emission = 520 – 620 nm). ERK1/2 phosphorylation is expressed as a percentage response to 10% (v/v) FBS (positive control).

**Inhibition of cAMP accumulation**

18 h after transfection, CHO-RXFP4 cells were trypsinized and seeded onto 96-well plates at a density of ~50,000 cells well per well and incubated for 6 – 8 h in complete media to allow cell adhesion. Cells were then incubated with PT, EcPltAB, or control (serum-free DMEM/F-12 medium) for 18 h. On the day of experiment, we changed the medium to Hanks’ balanced salt solution supplemented with 500 µM isobutylmethylxanthine, 5 mM HEPES, and 0.1% (w/v) BSA and equilibrated for 1 h at 37 °C. Cells were treated with INSIL5 (100 nM) for 15 min, followed by 30 min of forskolin stimulation and then lysed with 100% ice-cold ethanol. Once ethanol has evaporated, cAMP was resuspended in 100 µl of lysis buffer containing 0.3% (v/v) Tween 20, 5 mM HEPES, and 0.1% (w/v) BSA. The amount of cAMP generated by forskolin and its inhibition by INSIL5 was measured using a homogeneous time-resolved fluorescence resonance energy transfer LANCE cAMP kit (PerkinElmer Life Sciences) as per the manufacturer’s protocol.

**Author contributions**—D. R. L. wrote the manuscript, purified proteins, and obtained the EcPlt structures; M. T. T. and M. D. J. cloned constructs and performed the ADP-ribosylation assays; O. K. performed mass spectrometry experiments; D. G. M. and M. A. S. identified EcPltAB and performed the bioinformatics analysis; S. Y. A., J. C. P., J. R., M. A. S., and T. B. provided experimental design oversight and interpretation.

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