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Recent insights into *Pasteurella multocida* toxin and other G-protein-modulating bacterial toxins

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Abstract

Over the past few decades, our understanding of the bacterial protein toxins that modulate G proteins has advanced tremendously through extensive biochemical and structural analyses. This article provides an updated survey of the various toxins that target G proteins, ending with a focus on recent mechanistic insights in our understanding of the deamidating toxin family. The dermonecrotic toxin from *Pasteurella multocida* (PMT) was recently added to the list of toxins that disrupt G-protein signal transduction through selective deamidation of their targets. The C3 deamidase domain of PMT has no sequence similarity to the deamidase domains of the dermonecrotic toxins from *Escherichia coli* (cytotoxic necrotizing factor (CNF)1-3), *Yersinia* (CNFY) and *Bordetella* (dermonecrotic toxin). The structure of PMT-C3 belongs to a family of transglutaminase-like proteins, with active site Cys–His–Asp catalytic triads distinct from *E. coli* CNF1.

Keywords

cysteine protease; cytotoxic necrotizing factor; deamidation; dermonecrotic toxin; GTPase; heterotrimeric G proteins; *Pasteurella multocida* toxin; signal transduction; transglutamination

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Bacterial protein toxins comprise a formidable arsenal for modulating host–pathogen interactions. From extensive genetic and biochemical studies over the past few decades, coupled with the numerous crystal structures now available, we have made enormous progress in our understanding of toxin-mediated disease processes. Information gleaned from these studies has also enabled scientists to exploit many of them as selective and efficient tools in research applications to dissect signaling mechanisms within eukaryotic cells, and as therapeutic agents in clinical applications [1–9].

A number of these toxins share the common feature of being large multipartite enzymes (A–B toxins) or effector proteins (sometimes named exoenzymes), which catalyze reactions that

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can interfere with host cell signal transduction and physiological processes. The functional B parts of A–B toxins bind host cell receptors, and mediate entry into and traffic within the host cell, as well as subsequent delivery of the functional toxic A part into the host cell cytosol. The toxic effector proteins, on the other hand, do not have B parts but, instead, are delivered directly from the bacterial cell into the eukaryotic cell through specialized bacterial secretion systems, the best studied of which are the type III secretion system (T3SS) and type IV secretion system (T4SS). Many of these delivered toxic units are highly specialized enzymes that alter the activity of cellular target proteins, most often through covalent modification. The enzyme activities reported for these toxins include ADP ribosylation [10], glucosylation [11], DNA degradation [12], deadenylation [13], acetylation [14], protein phosphorylation/dephosphorylation [15], proteolysis [16], actin-crosslinking [17] and deamidation/transglutamination [18–20].

G proteins are the molecular targets for a large number of intracellularly acting toxins (Table 1). This article will begin with a brief overview of the different G-protein-targeting toxins, and then will hone in on recent structural and mechanistic advances that have been made regarding the deamidating toxin family, particularly in light of the determination that the structural fold and active site of the *Pasteurella multocida* toxin (PMT) deamidase is distinct from that of the cytotoxic necrotizing factor (CNF)-1-related family members [21].

G proteins as targets of bacterial toxins

G proteins are GTPases that bind and hydrolyze GTP and act as regulatory molecular switches in various signaling processes by cycling between an inactive GDP-bound state and an active GTP-bound state [22–25]. There are three large families of G proteins that serve as targets for toxins: small GTPases of 20–25 kDa, the 40–45-kDa α-subunits of heterotrimeric G proteins and the large (~100-kDa) multidomain elongation factors that regulate protein synthesis through their GTPase activity.

In their inactive GDP-bound state, small G proteins are often complexed with a guanine nucleotide dissociation inhibitor (GDI), which stabilizes the GDP–GTPase complex (Figure 1). Activation of the G protein occurs by exchange of the bound GDP with GTP through the interaction with guanine nucleotide exchange factors (GEFs). In the active GTP-bound state, small GTPases can interact with their target effector proteins and thereby modulate numerous downstream signaling processes involved in cytoskel-etal function, cell polarity, secretion, vesicle trafficking, gene transcription and cell cycle progression [26]. For instance, members of the Rho GTPase family RhoA, Rac and Cdc42, act to promote formation of stress fibers, lamellipodia and filopedia, respectively, whereas RhoD acts in opposition to RhoA to disassemble actin stress fibers.

Heterotrimeric G proteins, comprised of α, β and γ subunits, constitute a large family of GTPases that transduce extracellular hormonal signals from ligand-bound integral membrane receptors to eukaryotic effector proteins involved in various signal transduction pathways and metabolic processes [27]. The α subunits of heterotrimeric G proteins from invertebrates and vertebrates are distinguished into four main classes based on sequence and functional similarities: G_s (G_s, G_{olf} and G_{gus}), G_i ($G_{i1/i2/i3}$, $G_{oA/oB}$, $G_{t1/t2}$ and G_z), G_{12} (G_{12}

and G_{13}), and G_q (G_q, G_{11}, G_{14} and $G_{15/16}$) [28]. As shown in Figure 2, Gα subunits cycle between an inactive receptor-bound, Gβγ-complexed state, with GDP bound, and an active GTP-bound state, where the Gα subunit is dissociated from the activated ligand-bound receptor and the Gβγ subunits [27]. Each of the Gα subunits has its own downstream signaling effector protein(s) that it interacts with when in the GTP-bound state. The Gβγ subunit complex increases the binding affinity of the Gα subunit for the corresponding receptor, and also regulates its own set of effectors when dissociated from the Gα subunit.

G proteins have intrinsic GTPase activity that can convert the bound GTP back to GDP and, in doing so, return the G protein back to its inactive state. The intrinsic GTPase activity of most G proteins is low, ranging from 0.02 min^{-1} for Ras-like small GTPases to 2–5 min^{-1} for α subunits of heterotrimeric G proteins [23,24,29]. The Gα subunits have two conserved active-site residues, glutamine and arginine, which stabilize the transition state of the GTPase. Small GTPases also have analogous active-site glutamines, but the corresponding active-site arginine is absent, accounting for the relatively low intrinsic GTPase activity for the small GTPases. Other regulatory proteins, known as GTPase-activating proteins (GAPs), can help stimulate this intrinsic GTPase activity by as much as 1000-fold, often by supplying a catalytic arginine group, sometimes referred to as an arginine finger [30–34]. For heterotrimeric Gα proteins, GTP hydrolysis can be stimulated by regulator of G protein signaling (RGS) proteins, which bind the switch regions of Gα subunits and stabilize the GTPase transition state, but do so without providing an arginine finger [35].

Large, multidomain GTPases, such as the elongation factors involved in protein synthesis, have conserved active-site arginines, but the intrinsic GTPase activity is very low and only enhanced upon binding with the ribosome in the pretranslocation phase of the peptide elongation cycle (Figure 3) [36–40]. The precise mechanism for this ribosome-mediated stimulation is still unclear.

Within this framework, most bacterial toxins that act on G protein targets do so by locking them into either an inactive or an active state (Table 1). Consequently, many signaling pathways modulated by these G proteins have been elucidated through the selective action of bacterial toxins.

Bacterial toxins that modulate G-protein targets through ADP ribosylation

Diphtheria toxin from *Corynebacterium diphtheriae* and exotoxin A from *Pseudomonas aeruginosa* catalyze the ADP ribosylation of a unique, highly conserved post-translationally modified His residue at position 715 (diphthamide, 2-[3-carboxyamido-3-(trimethyammonio) propyl] histidine) on the large, multidomain GTPase elongation factor (EF)-2 of eukaryotes [41]. Precisely how this covalent modification alters EF-2 function is still unclear [36]. There is some evidence that ADP ribosylation of EF-2 does not interfere with GTP or GDP binding, but may inhibit the exchange of GDP with GTP [42]. His-715 is near the proposed interaction site of EF-2 with the codon–anticodon duplex, and it is thought to interfere with EF-2 binding to the ribosome in the pretranslocation phase of the peptide elongation cycle [36,42,43], which prevents formation of the high-affinity complex and stimulation of the GAP activity by the ribosome, effectively blocking protein synthesis and resulting in cell death. Archaea have a deamidated form of the diphthamide residue (known

as diphthine), which can also serve as a target for diphtheria toxin but at a 1000-fold slower reaction rate [44]. Prokaryotic elongation factors have a lysine at the analogous position instead and, thus, are not substrates for bacterial ADP ribosylating toxins.

Cholera toxin (CT) from *Vibrio cholerae* and pertussis toxin (PT) from *Bordetella pertussis* were the first toxins known to act on heterotrimeric G proteins [45,46], and have served as valuable tools for probing G-protein function in ligand–receptor-mediated signal transduction [8]. CT activates $G\alpha_s$ proteins, while PT inhibits $G\alpha_i$. proteins involved in coupling of hormone receptor-mediated regulation of adenylate cyclase signaling pathways [45]. CT and the closely related heat-labile enterotoxins (HLTs) from *Escherichia coli* catalyze the ADP ribosylation of an active site arginine (Arg-201) in $G\alpha_s$ subunits. This modification results in dissociation of the Gα subunit from the βγ subunits and locks the Gα subunit in an active state that can stimulate its downstream effectors [47]. PT catalyzes the ADP ribosylation of a Cys residue (Cys-352) four amino acids from the C-terminus of $G\alpha_i$. proteins. Other Gα proteins do not have a Cys at this position and are often referred to as PT-insensitive G proteins [48]. The preferred substrate for PT is the heterotrimeric form of the G_i protein [49]. ADP ribosylation of $G\alpha_i$ proteins by PT uncouples the $G\alpha_i$ protein interaction with, and activation by, the receptor, which, in turn, impedes GDP/GTP exchange and effectively locks the Gα subunit in its GDP-bound heterotrimeric form [50].

A number of ADP ribosylating toxins act on small GTPases of the Rho and Ras families to inactivate them [51]. These small GTPases modulate cellular processes, such as actin cytoskeletal organization and dynamics, membrane trafficking, cell proliferation and apoptosis [26,52]. The exoenzyme C3 from *Clostridium botulinum* specifically catalyzes the ADP ribosylation of an Asn residue (Asn-41) of Rho GTPases (RhoA, RhoB and RhoC, but not Rac, Cdc42 or Ras), inactivating the Rho proteins and causing depolymerization of actin filaments [53]. C3-like ADP ribosyltransferases are also produced by *Clostridium limosum* [54], *Staphylococcus aureus* [55,56] and *Bacillus cereus* [57]. ADP ribosylation of Rho at Asn-41 has little or no effect on GDP/GTP binding, or on the intrinsic GTPase activity, but appears to interfere with the activation of the G protein by its GEF, Lbc [58].

Bacterial toxins that modulate G-protein targets through glucosylation

RhoA and other related GTPases, such as Rac and Cdc42, are targets of the *Clostridium difficile* toxins TcdA and TcdB, *Clostridium sordellii* TcsH and TcsL, and *Clostridium novyi* Tcnα. These toxins, whose catalytic domains all belong structurally to the type A family of glycosyltransferases, inactivate their G-protein targets by monoglucosylation at a specific threonine residue (Thr-37 in Rho, Thr-35 in Rac and Cdc42), thereby inducing reorganization of actin and cell rounding [59,60]. In addition, TcdA and TcsL monoglucosylate the Ras-like proteins Rap1 and Rap2 [61,62]. With the exception of Tcnα, which also utilizes UDP-*N*-acetylglucosamine, all the other clostridial glucosyltransferases use UDP-glucose as a cosubstrate. Inactivation of the small GTPases by glucosylation prevents the G protein from interacting with its downstream effector proteins [58].

Bacterial toxins that modulate G-protein targets through proteolysis

The *Yersinia pseudotuberculosis* T3SS effector protein (YopT) and the related *Pseudomonas syringae* T3SS effector protein (Avr/PhpB) also inactivate the small GTPases RhoA, Rac and Cdc42, but they do so by proteolytic cleavage of a C-terminal peptide that contains a post-translationally modified Cys residue (with an isoprene moiety) [63]. Loss of this peptide prevents membrane localization of the G protein and uncouples downstream signaling.

Bacterial toxins that modulate G-protein targets through noncovalent interactions

Several T3SS effector proteins from *Salmonella* (SopE, SifA and SifB) [64,65], *Shigella* (IpgB1 and IpgB2) [66], *E. coli* (Map, EspM and EspT) [67–69], and *Citrobacter rodentium* (EspM and EspT) [67,68,70] belong to a family of proteins containing a WxxxE motif, which activate Rho GTPases through GEF-like mechanisms [71]. Although originally proposed to be Rho GTPase mimics [72], structural and biochemical evidence have shown that these WxxxE-motif-containing T3SS effector proteins are, indeed, Rho GEFs, with structurally similar GEF-like domains that selectively regulate RhoA, Rac1 or Cdc42 signaling pathways by interacting with the switch I and switch II regions of the GTPases [64,65,69,71]. By contrast, the GEF-like domain of the T4SS effector RalF from *Legionella pneumophila*, which subverts Arf GTPase signaling has no structural similarity with the WxxE-motif family of bacterial GEFs, but does have structural similarity to the GEF domain of the mammalian Dbl/Sec7 protein family of Rho GEFs [73].

Bacterial toxins that modulate multiple G-protein targets through multiple activity domains

Pseudomonas aeruginosa ExoS and ExoT are bifunctional cytotoxic T3SS effector proteins that share 76% protein sequence homology [74]. The N-terminal domain of both cytotoxins acts as a GAP for the Rho GTPases, Rho, Rac and Cdc42, by supplying a catalytic arginine residue (Arg-146 of ExoS) to stabilize the GTPase transition state [75,76], which results in actin depolymerization and interference with phagocytosis. Other T3SS effector proteins with similar GAP-like domains include the protein tyrosine phosphatase from *Salmonella enterica* serovar Typhimurium (SptP) [77,78] and the YopE protein from *Y. pseudotuberculosis* [79]. Although the primary amino acid sequences of the ExoS, SptP and YopE GAP domains have limited similarity, their crystal structures are strikingly similar [80]. Interestingly, while the RhoGAP domain of ExoS is a functional mimic of eukaryotic GAPs, their structures do not share any similarity [76]. The C-terminal domains of ExoS and ExoT both have ADP ribosyltransferase activity but, in contrast to their RhoGAP domains, the ADP ribosyltransferase domains do not have the same substrate specificity [74]. The C-terminal domain of ExoS catalyzes the ADP ribosylation of a number of host signaling proteins [81,82], including two arginine residues (Arg-41, Arg-128) of Ras GTPases [83], which uncouples mitogenic signal transduction by preventing activation of Ras by its GEF Cdc25 [84]. By contrast, the C-terminal domain of ExoT specifically ADP ribosylates Crk-I and Crk-II, which are Src homology 2–3 domain-containing adaptor proteins involved in regulation of focal adhesion and phagocytosis [85].

Another bifunctional T3SS effector protein has been identified from *Yersinia* – YpkA in *Y. pseudotuberculosis* and YopO in *Y. enterocolitica* [86]. YpkA has two domains that act

synergistically to disrupt host actin organization and prevent phagocytosis. The C-terminal domain is a Rho GDI that inhibits GDP/GTP exchange of small GTPases of the Rho family [87]. The N-terminal domain of YpkA is a serine/threonine protein kinase that phosphorylates $G\alpha_q$ at Ser-47, a key residue in the diphosphate-binding site of the GTPase domain and, thereby, blocks GDP/GTP binding and inhibits $G\alpha_q$ signaling [86].

Legionella pneumophila uses the bifunctional DrrA/SidM T4SS effector protein to activate and recruit the Golgi–endoplasmic reticulum (ER) vesicle-trafficking regulator Rab1 to the specialized *Legionella*-containing vacuole through both Rab1-specific GEF-like activity and GDI-displacement factor (GDF)-like activity [88,89]. DrrA/SidM has extensive interactions with the switch I and II regions of Rab1, which result in displacement of the switch I region [90]. In addition, *L. pneumophila* has another T4SS effector protein, LepB, which has Rab1 GAP-like activity that, in conjunction with DrrA/ SidM, functions in membrane cycling of Rab1 by promoting hydrolysis of GTP and release of Rab1 from the membrane [88].

Another family of large bacterial toxins with multiple activity domains is the multifunctional autoprocessing repeats of toxins (MARTX) of the *Vibrio, Aeromonas, Photorhabdus* and *Yersinia* genera [17]. The MARTX from *Vibrio cholerae* (Mr ~460 kDa), and the related one from *V. vulnificus*, cause the ‘rounding up’ of cells. One of the functional domains that these proteins possess is a Rho-inactivation domain (RID), which causes inactivation of Rho GTPases through a currently unknown mechanism [91], but these large proteins are comprised of several additional domains that contribute to cytotoxicity. The flanking N- and C-termini contain extensive repeat regions, which are involved in membrane pore formation and translocation of the multiple effector domains, including the RID, an actin-crosslinking domain (ACD) with sequence similarity to glutamine synthetases [92–94], an α-β hydrolase-like domain of unknown function, and an autocatalytic, inositol hexakisphosphate-dependent caspase-like cysteine protease domain (CPD) that processes the MARTX to release the effector domains into eukaryotic cells [95–97]. MARTX proteins illustrate how toxins can have multiple effects on multiple aspects of host cell processes, including G-protein function.

Bacterial toxins that modulate small G-protein targets through deamidation and/or transglutamination

In addition to inactivation of the G-protein targets, toxin-catalyzed modifications can also lead to activation. Modifications by the CNFs from *E. coli* and *Yersinia* (CNF1, CNF2, CNF3 and CNFY) and the dermonecrotic toxin (DNT) from *Bordetella* spp. activate the small GTPases, RhoA, Rac and Cdc42, but not RhoD [19]. The switch I and switch II regions of Rho proteins are involved in protein–protein interactions between GTPases and their effectors. The CNFs modify Rho proteins by deamidation of a specific Gln residue, Gln-63 of RhoA [98–100] and Gln-61 of Rac and Cdc42 [101], located in the switch II region, while CNFY appears to have more stringent substrate specificity for RhoA and does not modify Rac or Cdc42 [102]. CNF1 has also been shown to transglutaminate RhoA at the same residue (Gln-63), although to a lesser extent [103]. DNT, on the other hand, activates Rho proteins primarily through transglutamination of the corresponding residues (Gln-63/61) [104–106], with putrescine, spermidine and spermine serving as the *in vivo*

cosubstrates for the transglutamination reaction [107,108]. Modeling of the DNT active site based on the structure for CNF1 suggests that this preference may stem from DNT having a substantial negative charge in the active-site pocket, which may be able to accommodate positively charged primary amines [109]. Both GDP-bound RhoA and GTP-bound RhoA can serve as substrates for CNF1, but DNT prefers GDP-bound RhoA [106]. The resulting modifications inhibit the intrinsic and GAP-stimulated GTPase activity of the targets, resulting in constitutive activation. Interestingly, a Glu residue (Glu-64 in RhoA) adjacent to the target Gln (Gln-63) appears to be critical for substrate recognition by the toxins; indeed, exchange of the equivalent residues in RhoD (Gln-75, Asp-76) to Gln–Glu converted RhoD into a substrate for CNF1 and DNT [110].

Pasteurella multocida toxin: a bacterial toxin that modulates heterotrimeric G proteins through deamidation

The $G\alpha_q$ family of $G\alpha$ subunits were first distinguished as the class of heterotrimeric G proteins that mediated activation of phospholipase Cβ (PLCβ) signaling pathways that were resistant to PT treatment [48,111–113], and for years there were no biochemical tools for studying the role of these G_q proteins in mitogenic and calcium signal-transduction pathways. This changed dramatically with the discovery that the *Pasteurella multocida* dermonecrotic toxin (PMT) strongly stimulates, but subsequently uncouples, PLCβ signal transduction through its action on the $G\alpha_q$ subunit, but not the closely related $G\alpha_{11}$ subunit [9,114,115]. PMT-mediated activation of $G\alpha_q$ leads to stimulation of calcium signaling through PLCβ hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to release inositol trisphosphate (IP_3) and diacylglycerol (DAG) [114–121], activation of mitogenic signaling through MAPK and STAT protein phosphorylation [122–126], and Rho-dependent actin cytoskeletal rearrangements [126–132].

Exposure to PMT directly facilitated $G\alpha_q$ -mediated activation of PLCβ1 and, to a lesser extent, PLCβ3, but not PLCβ2 [114], in-keeping with known cellular responses elicited by $G\alpha_q$ -coupled receptors [111,112]. This strong initial response was potentiated by release of the $G\alpha_q$ subunit from the heterotrimeric complex through either PT-mediated sequestration of the $G\beta\gamma$ subunits or through dissociation of the $G\alpha$ subunit from $G\beta\gamma$ by using antibodies against the Gβ subunit [114]. PMT action on $G\alpha_q$ is irreversible and persistent [114,133] and independent of interaction with G-protein-coupled receptors [133]. Furthermore, overexpression of $G\alpha_q$ enhanced the PMT-induced response, while decreased expression of $G\alpha_q$ or treatment with GDPβS, a known inhibitor of $G\alpha$ signaling, blocked the PMT-induced response [114], suggesting that the monomeric form of $G\alpha_q$ is the preferred substrate of PMT. Recently, the biochemical activity of PMT was determined to be through deamidation of $G\alpha_q$ at Gln-209 [21]. Interestingly, Gln-209 is functionally equivalent to Gln-63 of the small GTPase RhoA, which serves as the target of the CNFs and DNT.

Pasteurella multocida dermonecrotic toxin is also a potent activator and subsequent uncoupler of $G\alpha_i$. signaling, converting the G protein into a form that is no longer sensitive to PT treatment [134]. Treatment of intact wild-type, $G\alpha_{q/11}$-deficient or $G\alpha_{12/13}$-deficient mouse embryonic fibroblasts with PMT leads to inhibition of isoproterenol and forskolin-mediated stimulation of adenylate cyclase activity, as well as cAMP accumulation through

G_s -coupled receptors, while enhancing the inhibition of cAMP accumulation by lysophosphatidic acid (LPA) through G_i-coupled receptors. PT treatment blocked LPA-mediated inhibition of cAMP accumulation, yet was unable to block PMT-mediated activation of $G\alpha_i$. or inhibition of cAMP accumulation. Moreover, pretreatment of cells with PMT prevented PT-induced ADP ribosylation of $G\alpha_{i2}$, in keeping with the proposed model where PMT acts on the monomeric $G\alpha$ subunit to irreversibly convert it into an active state [114]. This effectively shifts the equilibrium to dissociate the heterotrimeric complex and release the $G\beta\gamma$ subunits [135], which can then interact with their downstream effector proteins. Since the preferred substrate for PT is the heterotrimeric G protein, and not the monomeric $G\alpha$ subunit [136], PMT deamidation of the $G\alpha_{i2}$ subunit at Gln-205 converts it into a form that is no longer a substrate for PT [21].

Identification of $G\alpha_{i2}$ as a substrate for PMT also enabled further study of the effect of PMT on the GTPase activity of the $G\alpha_i$. subunit. PMT treatment of cells reduced both basal and LPA-induced hydrolysis of GTP by the $G\alpha_i$. protein in membrane preparations [134]. A similar effect was observed with the use of the wasp venom peptide mastoparan, a widely used receptor-independent activator of $G\alpha_i$, suggesting that PMT-mediated activation of $G\alpha_i$ and subsequent inhibition of adenylate cyclase may be caused by PMT-mediated inhibition of the intrinsic GTPase activity of $G\alpha_i$. However, PMT also inhibited LPA-receptor-stimulated binding of GTPγS to $G\alpha_i$. [134]. This finding supports the model for PMT action, where PMT first locks the monomeric $G\alpha_i$ subunit in its active form through deamidation, which inhibits its GTPase activity [21]. This action prevents reassociation of the $G\alpha_i$. subunit with the $G\beta\gamma$ subunits, as evidenced by the failure of PMT-exposed $G\alpha_{i2}$ to bind to $G\beta\gamma$ and serve as a substrate for PT [21], and essentially leads to the functional uncoupling of the G protein from its receptor, similar to what was observed for PMT action on $G\alpha_q$ [114].

In addition to the activation of $G\alpha_q$ and $G\alpha_i$ proteins, PMT activates $G\alpha_{12/13}$ signaling pathways [137], resulting in formation of actin stress fibers and assembly of focal adhesions through indirect activation of RhoA mediated by the regulator of G-protein signalling (RGS) domains of Rho GEFs, such as LARG, p115-RhoGEF, PDZ-RhoGEF or Dbl [138–141]. $G\alpha_{12}$ and $G\alpha_{13}$ share 67% sequence identity, except for their N-terminal 30 residues, which only share 16% identity and confer receptor specificity [142]. In GG$\alpha_{q/11}$-deficient fibroblasts, RhoA activation by PMT was inhibited by dominant-negative $G\alpha_{13}$, whereas in $G\alpha_{12/13}$ -deficient cells, RhoA activation by PMT could be reconstituted by infection with retrovirus encoding $G\alpha_{13}$ [137]. Although PMT-mediated activation of $G\alpha_{12}$ signaling was not tested in this study and direct deamidation of $G\alpha_{12}$ and $G\alpha_{13}$ by PMT has not yet been demonstrated, both $G\alpha_{12}$ and $G\alpha_{13}$ have analogous switch II Gln residues (Gln-229) that could serve as PMT targets for deamidation. However, it should be noted that $G\alpha_q$ and $G\alpha_{11}$ share even greater homology (88% sequence identity) with each other, including the switch II Gln-209 residue [113], but only $G\alpha_q$ is a substrate for PMT [21,115]. The reason for this difference in substrate specificity between $G\alpha_q$ and $G\alpha_{11}$ is not known; however, exchange of two residues (Glu-105 and Asn-109) in the helical domain of $G\alpha_{11}$ with the corresponding His residues of $G\alpha_q$ rendered the mutant $G\alpha_{11}$ now capable of mediating PMT-induced activation of PLCβ in $G\alpha_{q/11}$-deficient fibroblasts [143]. It is not yet known whether this differential interaction is due to differences in PMT substrate recognition of

$G\alpha_q$ versus $G\alpha_{11}$ or due to differential interaction of the $G\alpha_q$ and $G\alpha_{11}$ proteins with the PLCβ1 effector protein.

Structural comparisons

PMT & related dermonecrotic toxins

When the sequences of PMT and the related CNFs and DNT were first examined, there were no matches found in the databases other than with each other and, thus, only biochemical and structural analyses provided insights into their functional organization. The receptor-binding domain is located in the N-terminus of each of these toxins [144–146], whereas the intracellular activity domain resides in the C-terminus [109,145–149]. The crystal structure of the catalytic domain of CNF1 (PDB 1HQ0) has been solved [109], and since the CNFs and DNT share significant sequence similarities (27–32%) in their C-terminal domains (residues 720–1014 in the CNFs, 1176–1464 in DNT), as well as similar target substrates and catalytic activities, it is presumed that their activity domains also have similar overall structures. Indeed, the Cys and His residues located in a putative active-site pocket (Figure 4) are not only conserved in all members of the CNF/DNT family (Cys-866 and His-881 in CNF1, and Cys-1305 and His-1320 in DNT), but are also essential for catalytic activity [103].

Although the CNFs and DNT share limited sequence similarity in their N-terminal receptor-binding and translocation domains with PMT, there is no discernable sequence similarity of their C-terminal catalytic domains with the C-terminal intracellular activity domain of PMT. This was confirmed when the crystal structure of a biologically active C-terminal fragment of PMT consisting of residues 569–1285 (PDB 2EBF) became available [148]. The structure of this fragment revealed three distinct domains: a C1 domain (residues 575–719) with sequence, structural and functional similarity to the membrane-targeting domain of the clostridial toxin TcdB [150]; a C2 domain (residues 720–1104) of currently unknown function, and a C3 domain (residues 1105–1285), with a papain-like cysteine protease structural fold. The PMT-C3 domain was subsequently demonstrated to harbor the minimal domain responsible for toxin-mediated activation of calcium and mitogenic signaling [147]. Disruption of the disulfide bond between Cys-1159 and Cys-1165 in the C3 domain through mutation of Cys-1159 to Ser exposed an active-site Cys–His–Asp triad (Cys-1165, His-1205, Asp-1220) [148]. This finding agreed with earlier studies that demonstrated the importance of Cys-1165 [151] and His-1205 [152] in the biological activity of PMT. However, other than the presence of active-site Cys and His residues, there was no indication that the catalytic activity of PMT was like that of the CNF/DNT family, since the protein folds were quite different (compare Figure 4A & B), and even the positioning of the active-site Cys and His groups were different.

Toxin G-protein deamidase/transglutaminase domains with other deamidases/ transglutaminases

The Cys–His–Asp triad is commonly involved in catalysis of two types of reactions: acyl hydrolysis (e.g., protease activity) or acyl transfer (e.g., transglutaminase activity). Based on the similarity of the Cys–His–Asp triad in PMT with that of papain, it was proposed that

PMT might act as a cysteine protease [148]. However, transglutaminases (TGases), which exchange the amine group of the side-chain γ-carboxyamide of glutamine with the primary amine group of another molecule, have structural folds with active-site Cys–His–Asp triads similar to that of papain-like cysteine proteases [153]. In the absence of a primary amine-containing substrate, TGases can catalyze the hydrolysis of the γ-carboxyamide group of Gln residues, resulting in deamidation. Deamidases, which convert the γ-carboxyamide group to a carboxylate, are closely related to TGases. Thus, the finding that the C-3 domain of PMT has G-protein Gln-deamidase activity [21] is consistent with its structure [148].

The structural fold of the PMT-C3 deamidase domain belongs to a family of structural folds that are closely related to mammalian and some bacterial TGases (Figure 4), typified by the human blood clotting factor XIII (PDB 1FIE) [154], fish-derived TGase from red sea bream (PDB 1G0D) [155], putative TGase-like cysteine protease from *Cytophaga hutchinsonnii* (PDB 3ISR), and the protein glutaminase from *Chryseobacterium proteolyticum* (PDB 2ZK9) [156]. The PMT-C3 core structural fold, similar to that of the other TGases, also bears similarity to the arylamine *N*-acetyltransferase (NAT) from *Salmonella enterica* serovar Typhimurium (PDB 1E2T) [157]. The active-site Cys–His–Asp triad is nearly superimposable for all five of these structures (Figure 5). The overall PMT-C3 structure most closely resembles that of the protein glutaminase from *Chryseobacterium*, with both the catalytic triad and the active-site cores clearly overlapping (Figure 4B). Interestingly, the structural fold of the microbial TGase from *Streptomyces mobaraensis* (PDB 1IU4) does not resemble the TGase-like PMT fold [158,159]. While still possessing a Cys–Asp–His triad at the active site, these catalytic residues in the *Streptomyces* TGase (Cys-64, Asp-255 and His-274) do not have the same geometry at the active site found for the PMT-C3 family.

A striking finding about the fish TGase and human factor XIII, in comparison with PMT-C3, is that the active sites of these proteins possess an additional Cys residue (Cys-333 in fish TGase and Cys-374 in factor XIII), which is separated from the catalytic Cys of the triad by an active site Tyr residue (Tyr-515 in fish TGase and Tyr-560 in factor XIII) [155]. It was proposed that the Tyr side chain sterically prevents disulfide bond formation, and subsequent inactivation in these proteins. Similar to these proteins, PMT also has another Cys residue (Cys-1159) near the catalytic Cys-1165, with which it does form a disulfide bond. It is interesting to speculate that reduction of the disulfide bond in PMT might occur prior to or during interaction with its substrate proteins.

In contrast to the TGase-like family of structural folds to which PMT belongs, the protein fold of the CNF1 catalytic domain appears to be unique to the CNF/DNT family of deamidating/transglutaminating toxins [109], as well as a few other bacterial proteins. It has been suggested that the CNF1 fold resembles that of the chemoreceptor-modifying deamidases, such as CheD (PDB 2F9Z) from *Thermotoga maritima* [160]. A similar structural topology has also been found in several proteins of unknown function, including YfiH from *Shigella flexneri* (PDB 1XAF and 1U05) [161], YlmD from *Bacillus stearothermophilus* (PDB 1T8H), protein CC_0490 from *Caulobacter crescentus* (PDB 1XFJ), and the YfiH-like protein from *Salmonella enterica* serovar Typhi (PDB 1RW0). Unlike CheD and the CNF/DNT toxins, these YfiH-like proteins have a second active-site His residue (His-71), in addition to the Cys–His dyad (Cys-107 and His-124 in YfiH), which

acts to coordinate zinc in some of the structures. The role that this zinc plays in the function of these proteins is currently unknown.

Future perspective

Increasing structural data, backed by extensive biochemical studies, are providing new insights into the range of biological functions that can be manipulated by bacterial protein toxins. The consequent depth and scope of our knowledge of their structure and function has enabled us to gain a better picture of the role that these toxins play in host–microbe interactions and bacterial pathogenesis. Indeed, we are now poised to begin contemplating ways to counteract the deleterious effects of these toxins on the host such that we can develop postexposure antitoxin therapeutics. We are even starting to get glimpses of potential roles for these toxins in long-term sequelae to bacterial infection, such as possible involvement of some of the mitogenic toxins, such as PMT in cancer onset or progression [162].

Importantly, our emerging understanding of their biochemical activities and mechanisms of action at the molecular level has been invaluable in making these toxins available as powerful tools to study and manipulate the myriad cellular signaling pathways modulated by G proteins. With the addition of PMT to CT and PT as selective molecular tools for studying heterotrimeric G-protein signaling, we can now begin to discern the functions of the different heterotrimeric G proteins in signal transduction and physiological processes. It remains to be determined which of the other G-protein α-subunits might also be targets for deamidation by PMT, and what the substrate recognition determinants are that discriminate one target protein from another. The consequences to the toxin-modified G-protein molecule also remain unclear.

Although often deleterious to the infected host, a number of these toxins are already beginning to be exploited for beneficial medical applications, owing to the unique selectivity and potency of their activities [2]. Clinical trials are already underway for incorporation of DT, CT, PT, CNF and anthrax toxin into vaccines as adjuvants or as antigen-delivery vehicles. DT, ExoA and several other toxins are being tested as immunotoxins in cancer treatments. A number of toxins, such as CNF, PT, anthrax toxin and botulinum neurotoxin, are being developed as alternative biomedical therapeutics and cosmetics. Further advances will continue to be made as we determine cellular uptake and intracellular targeting mechanisms, decipher the determinants of toxin–substrate specificity, and differentiate the various consequences of toxin action on downstream signaling and cellular function.

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Executive summary

G proteins as targets of bacterial toxins

- Many bacterial toxins target regulatory G proteins, and act at different points in the GTPase cycle to disrupt G-protein signal transduction.

Bacterial toxins that modulate G-protein targets through ADP ribosylation

- Diphtheria toxin (DT) and *Pseudomonas* ExoA ADP ribosylate the large GTPase elongation factor (EF)-2 and block eukaryotic protein synthesis.
- Cholera toxin (CT) and pertussis toxin (PT) ADP ribosylate heterotrimeric G proteins involved in hormone receptor-mediated signal transduction.
- Clostridial C3 toxin ADP ribosylates and inactivates small Rho GTPases.

Bacterial toxins that modulate G-protein targets through glucosylation

- The large clostridial toxins inactivate small Rho GTPases through monoglucosylation.

Bacterial toxins that modulate G-protein targets through proteolysis

- Some type III secretion system (T3SS) effector proteins (YopT from *Yersinia* and Avr/PhpB from *Pseudomonas*) cleave and inactivate small Rho GTPases to prevent membrane localization.

Bacterial toxins that modulate G-protein targets through noncovalent interactions

- Some T3SS (*Salmonella* SopE, SifA and SifB; *Shigella* IpgB1 and lpgB2; *Escherichia coli* Map, EspM and EspT; and *Citrobacter* EspM and EspT) and T4SS (*Legionella* RalF) effector proteins are guanine nucleotide exchange factors (GEFs) that activate Rho GTPases.

Bacterial toxins that modulate multiple G-protein targets through multiple activity domains

- Some toxins (*Pseudomonas* ExoS and ExoT, *Salmonella* SptP, *Yersinia* YopE, YkpA/YopO, and *Vibrio* MARTX) have multiple domains with different intracellular activities that modulate small GTPases.

Bacterial toxins that modulate G-protein targets through deamidation &/or transglutamination

- Deamidation (*E. coli* and *Yersinia* cytotoxic necrotizing factor [CNF]s) or transglutamination (*Bordetella* dermonecrotic toxin [DNT]) of a specific Gln residue (Gln-63 in RhoA) inhibits intrinsic and GAP-stimulated Rho GTPase activity, resulting in constitutive activation.
- *Pasteurella multocida* toxin (PMT) deamidates heterotrimeric G proteins, $G\alpha_q$ $G\alpha_{i2}$ and $G\alpha_{13}$, and possibly other members of the G_q family at a specific Gln residue (Gln-209 in $G\alpha_q$, Gln-205 in $G\alpha_{i2}$), which results in

initial stimulation of G-protein-mediated signaling, followed by uncoupling of the signal transduction.

Structural comparison of PMT & related dermonecrotic toxins

- PMT shares limited sequence similarity with the CNFs or DNT in their N-terminal receptor-binding and translocation domains.
- PMT shares no sequence or structural similarity with the CNFs or DNT in their C-terminal activity domains (residues 1105–1285 in PMT-C3, 720–1014 in the CNFs, 1176–1464 in DNT).
- The structure of PMT (575–1285) revealed three domains: C1 membrane-localization domain, C2 domain of unknown function, and C3 domain with cysteine protease-like Cys–His–Asp catalytic triad.

Structural comparison of toxin G-protein deamidase/transglutaminase domains with other deamidases/transglutaminases

- The structure of the PMT-C3 domain belongs to a family of transglutaminases (TGases; human factor XIII, fish TGase, *Chryseobacterium* protein glutaminase, *Salmonella* arylamine *N*-acetyltransferase and a putative *Cytophaga* cysteine protease).
- The PMT-C3 structural fold has no similarity to the CNF/DNT family of deamidases (*Thermotoga* CheD deamidase; *Shigella*, *Bacillus*, *Caulobacter*, and *Salmonella* YfiH-like proteins and *Streptomyces* TGase).

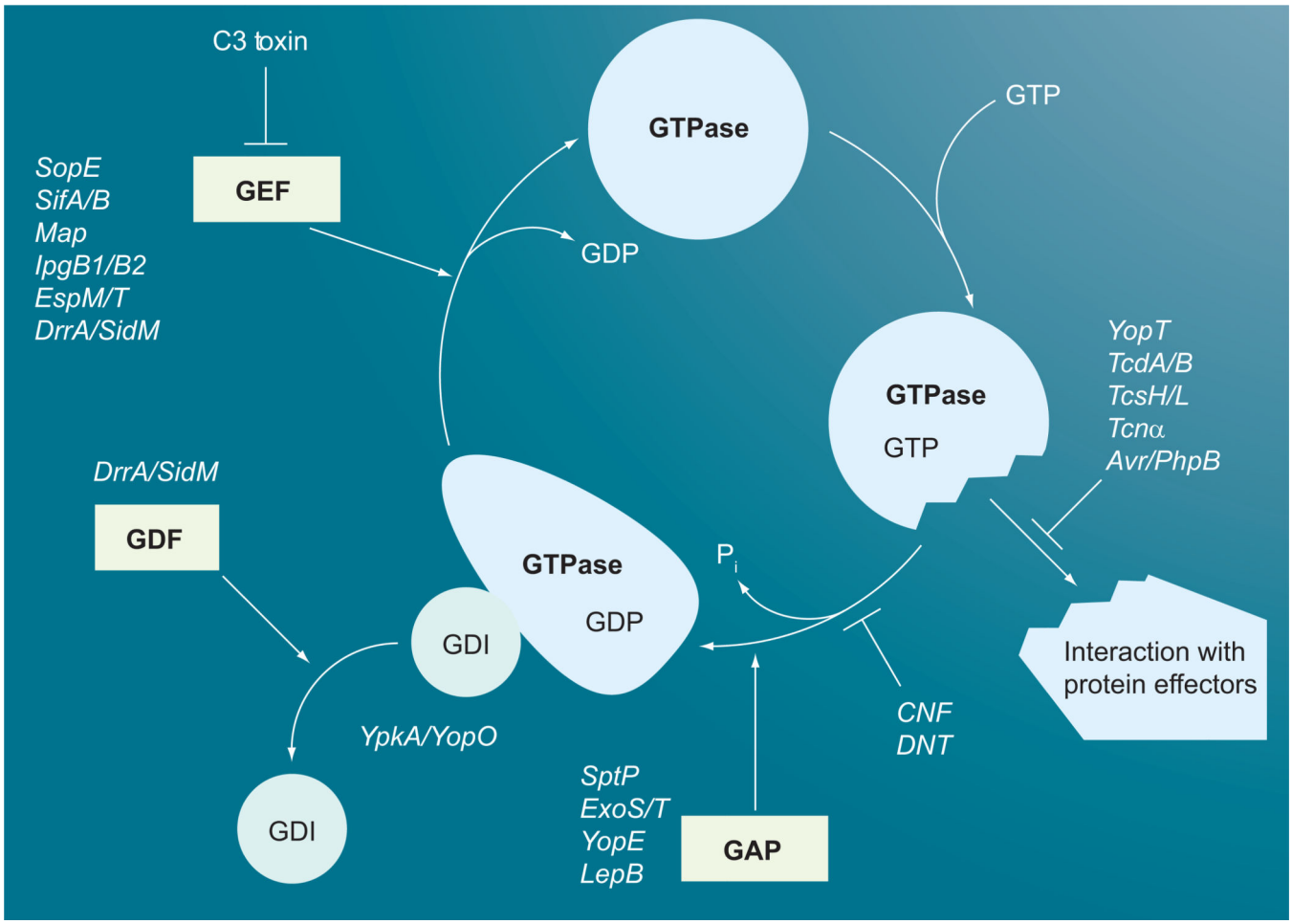

Figure 1. GTPase cycle of small G proteins and points of toxin interactions

Small GTPase binds GDI in the inactive GDP-bound form. GEF facilitates the release of GDI and GDP and the GTPase then binds GTP. The active GTP-bound form interacts with its downstream effectors. Subsequent interaction with GAP stimulates the hydrolysis of GTP to GDP, which converts the GTPase back into its inactive form. Large clostridial toxins (TcdA, TcdB, TcsH, TcsL and Tcnα), YopT and Avr/PhpB interfere with the GTPase interaction with effectors. CNF and DNT block GTPase activity, while the type III secretion system (T3SS) effectors SptP, ExoS, ExoT and YopE, and the T4SS effector LepB, act as GAPs to stimulate GTP hydrolysis. The T3SS effector YpkA/YopO acts as a GDI to prevent release of GDP. The clostridial C3 toxin blocks GEF interaction with the G protein, while the T3SS effectors SopE, SifA, SifB, Map, EspM, EspT, IpgB1 and lpgB2 act as GEFs. The T4SS effector DrrA/SidM acts as both a GEF and a GDF.

CNF: Cytotoxic necrotizing factor; DNT: Dermonecrotic toxin; GAP: GTPase activating protein; GDI: Guanine nucleotide dissociation inhibitor; GEF: Guanine nucleotide exchange factor.

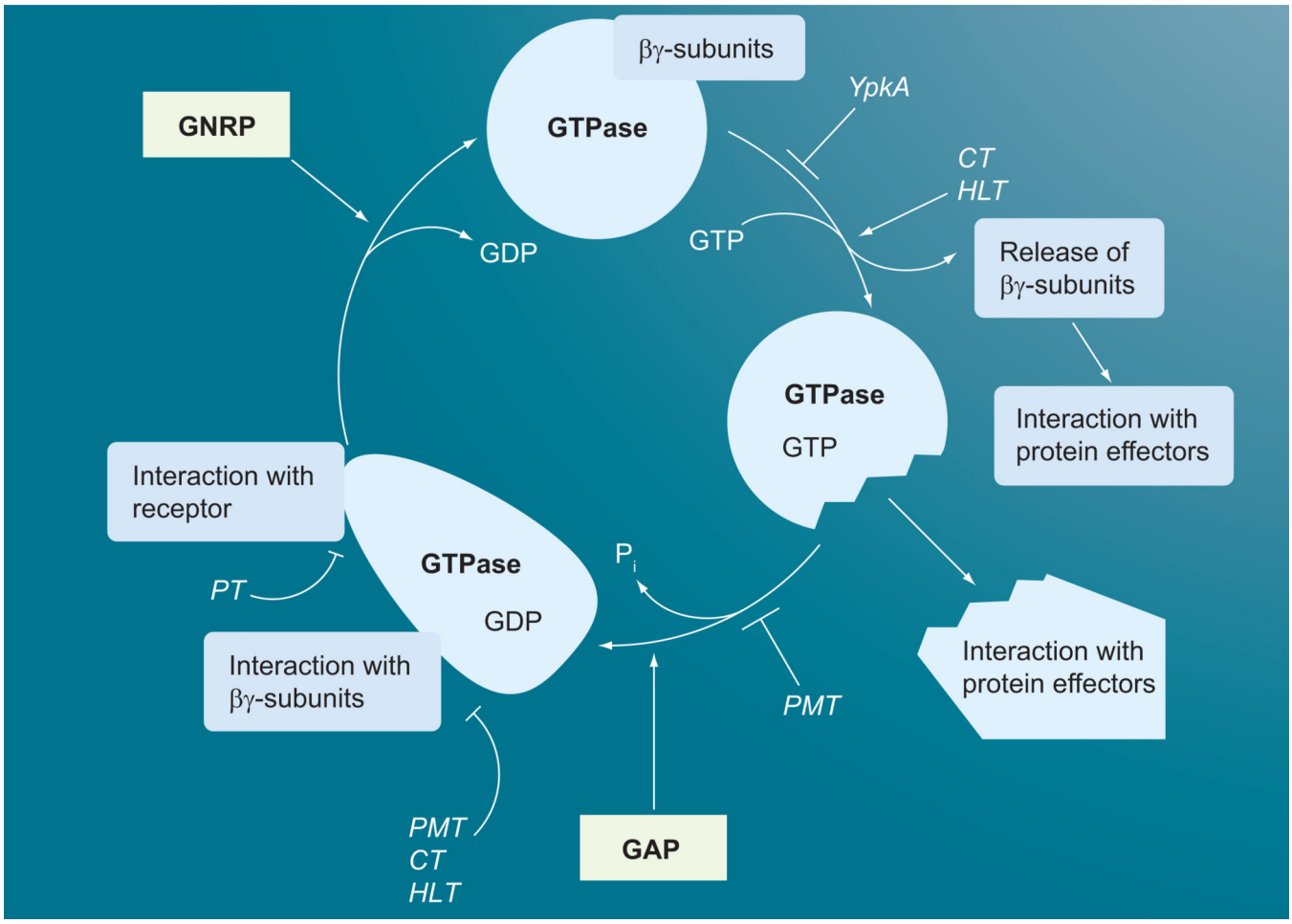

Figure 2. GTPase cycle of heterotrimeric G proteins and points of toxin interactions

Heterotrimeric GTPase α subunit binds βγ subunits in the inactive GDP-bound form. The G-protein-coupled receptor bound to its ligand acts as GNRP to stimulate the release of GDP and the α subunit then binds GTP and the βγ subunits dissociate to interact with their downstream effectors. The dissociated active GTP-bound α subunit then interacts with its downstream effectors. PMT, CT and HLT lock the α subunit in its active form and prevent interaction with the βγ subunits and the receptor. PT locks the G protein in its heterotrimeric inactive form and prevents its interaction with the receptor. YpkA prevents GDP/GTP binding to the α subunit.

CT: Cholera toxin; GNRP: Guanine nucleotide release protein; HLT: Heat-labile enterotoxins; PMT: *Pasteurella multocida* toxin; PT: Pertussis toxin.

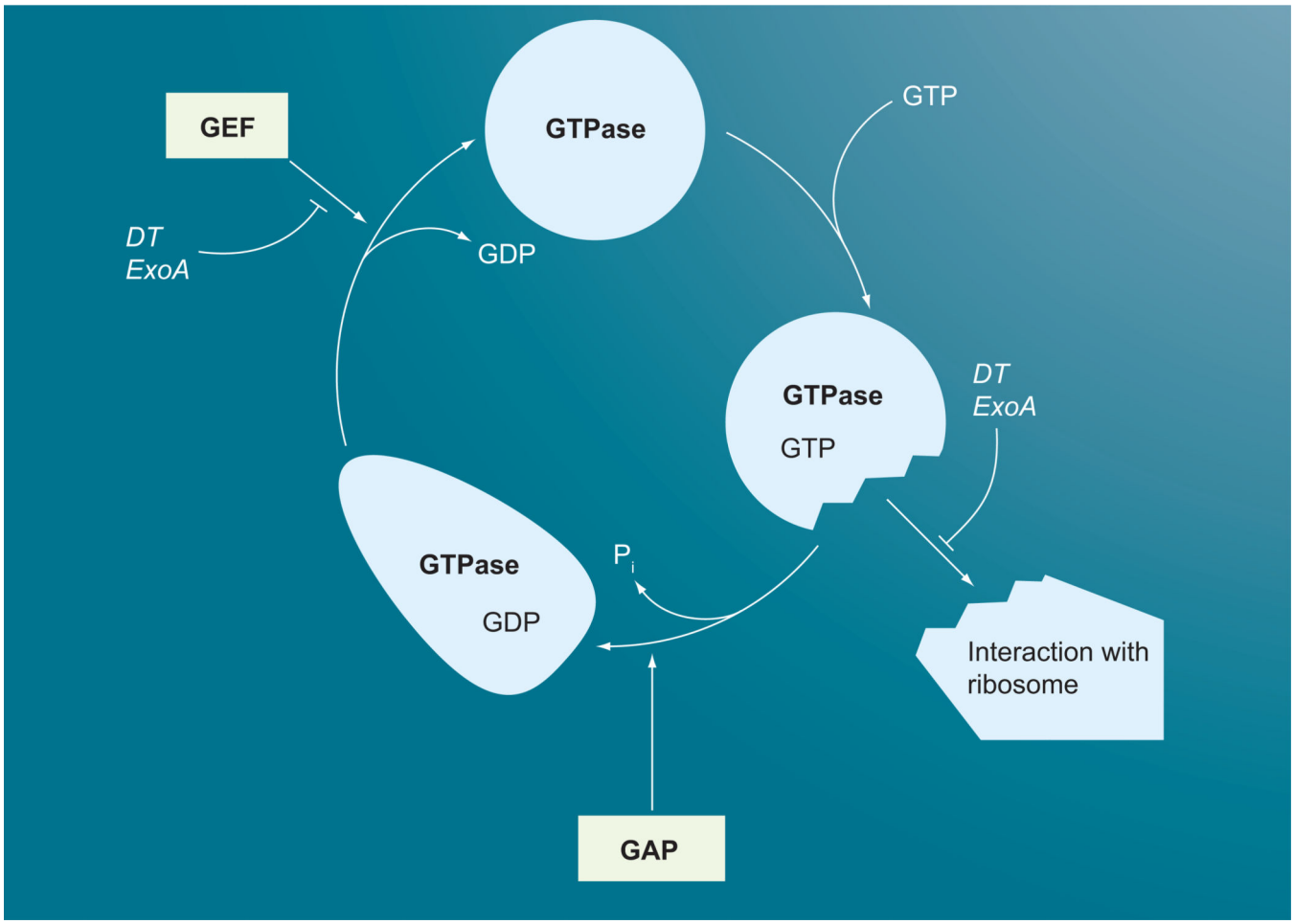

Figure 3. GTPase cycle of large G proteins and points of toxin interactions

ADP ribosylation of elongation factor (EF)-2 by DT or ExoA blocks interaction of GTP-bound EF-2 with the ribosome in the pretranslocation phase of the peptideelongation cycle, which prevents formation of the high-affinity complex and stimulation of the GAP activity of EF-2 by the ribosome. ADP ribosylation of EF-2 does not interfere with GTP or GDP binding to EF-2, but may inhibit the exchange of GDP with GTP.

DT: Diphtheria toxin; GAP: GTPase activating protein; GEF: Guanine nucleotide exchange factor.

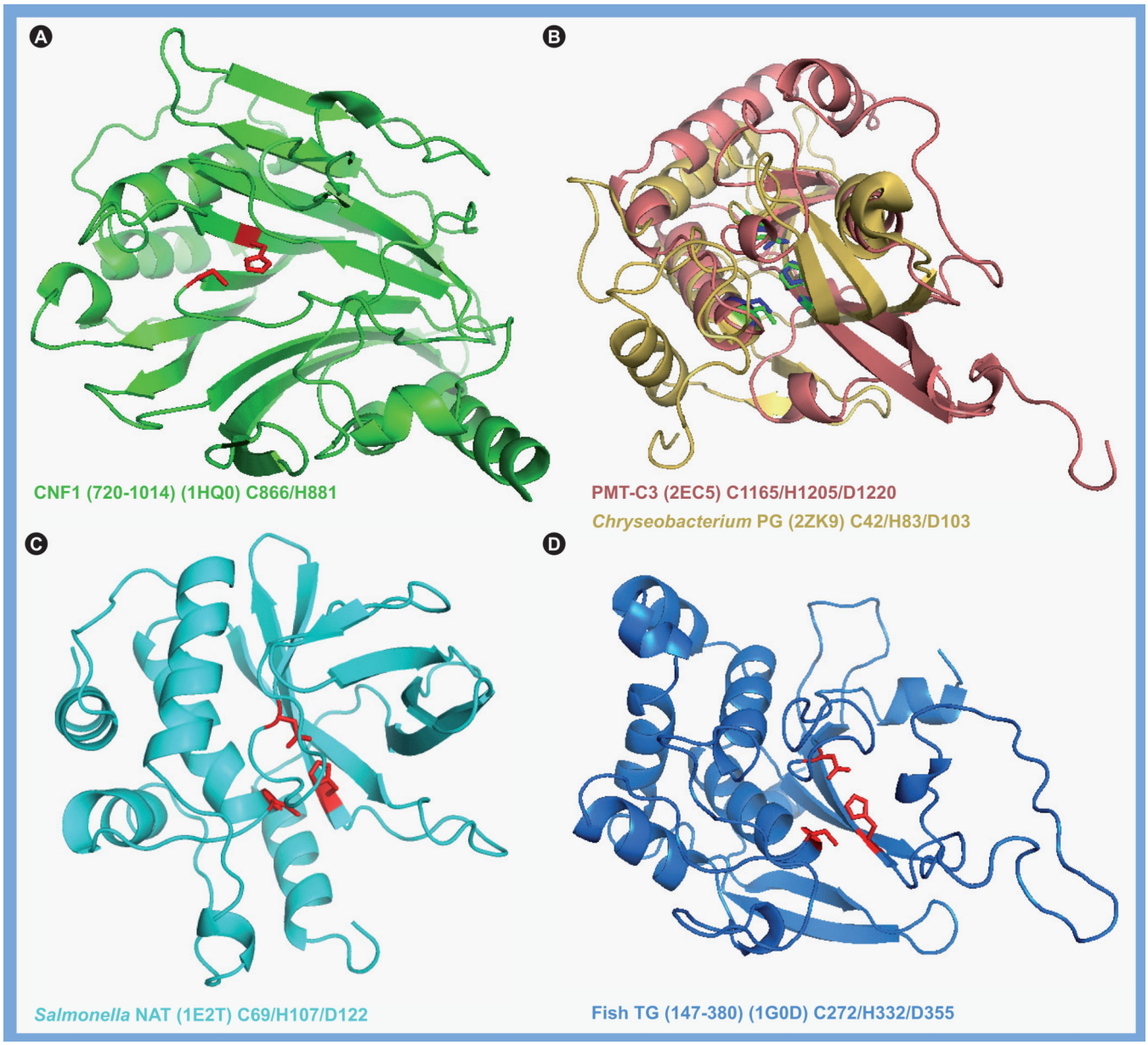

Figure 4. Structures of toxin-like deamidase/transglutaminase domains

Shown are the structural folds of the catalytic domains of representative members of the cytotoxic necrotizing factor/dermonecrotic toxin-like family and the PMT-like family of deamidases/ TGases, with the respective active site His–Cys dyad or His–Cys–Asp triad indicated. **(A)** Catalytic domain of CNF1 (PDB 1HQ0), residues 720–1014 shown in green, with the Cys-866 and His-881 shown in red. **(B)** Superimposition of the catalytic domains of PMT (PDB 2EC5), residues 1105–1285 shown in pink, with Cys-1165, His-1205 and Asp-1220 shown in blue, and the protein glutaminase from *Chryseobacterium proteolyticum* (PDB 2ZK9), shown in yellow, with Cys-42, His-83, and Asp-103 shown in green. **(C)** Catalytic domain of the arylamine *N*-acetyltransferase from *Salmonella enterica* serovar Typhimurium (PDB 1E2T), residues 1–197 shown in cyan, with the Cys-69, His-107, and

Asp-122 shown in red. **(D)** Catalytic domain of the fish-derived TGase from red sea bream (PDB 1G0D), residues 147–380 shown in blue, with Cys-272, His-332 and Asp-355 shown in red.

Images were generated with PyMOL using the indicated PDB data files.

CNF: Cytotoxic necrotizing factor; NAT: *N*-acetyltransferase; PDB: Protein Data Bank; PMT: *Pasteurella multocida* toxin; TGase: Transglutaminase.

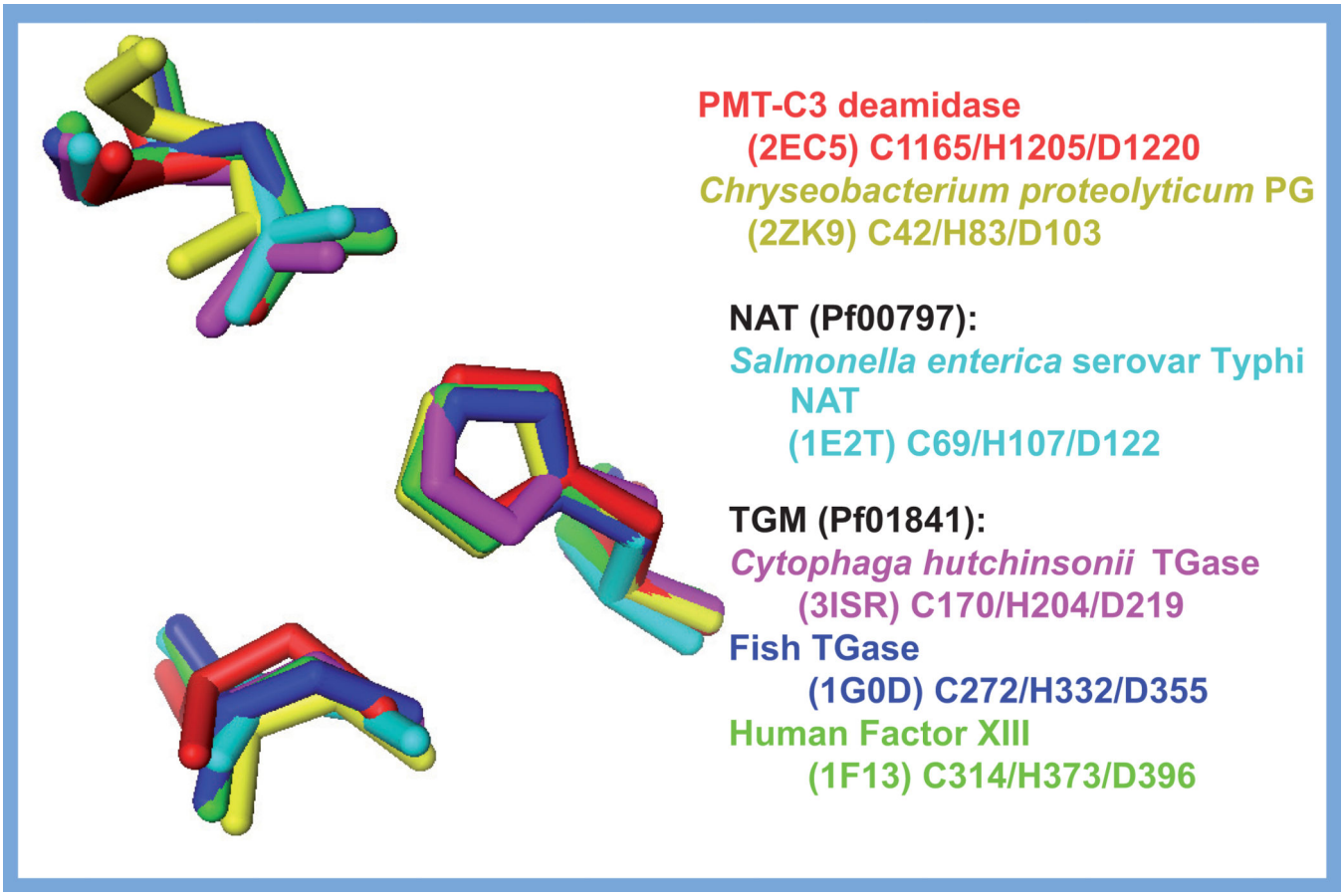

Figure 5. Comparison of the active-site catalytic triads of the *Pasteurella multocida* toxin-like deamidases/transglutaminases

Superimposed images of the active site Cys, His and Asp side chains of the catalytic triads from: PMT-C3 (PDB 2EC5) in red, *Chryseobacterium* protein glutatminase (PDB 2ZK9) in yellow, *Salmonella* NAT (PDB 1E2T) in light blue, *Cytophaga hutchlnsonll* TGase (PDB 3ISR) in magenta, fish-derived TGase (PDB 1G0D) in purple and human factor XIII (PDB 1F13) in green.

Images were generated with PyMOL using the indicated PDB data files.

NAT: *N*-acetyltransferase; PDB: Protein Data Bank; PMT: *Pasteurella multocida* toxin; TGase: Transglutaminases;

Table 1

G proteins and their modulating toxins.

G-protein targets	Modulating toxins	Modification	Effect on G protein	Ref.
Small GTPases				
RhoA, Rac1,Cdc42	TcdA, TcdB, TcsH, TcsL, Tcnα	Monoglucosylation	Inactivation	[59,60]
Rap1, Rap2	TcdA, TcsL	Monoglucosylation	Inactivation	[61,62]
RhoA, RhoB, RhoC	clostridial C3	ADP-ribosylation	Inactivation	[51]
Ras	ExoS	ADP-ribosylation	Inactivation	[79–82]
RhoA, Rac1,Cdc42	ExoS, ExoT, SptP, YopE	GAP-like activity	Inactivation	[72–74]
Rab1	LepB	GAP-like activity	Inactivation	[86]
RhoA, Rac1	YpkA, YopO	GDI-like activity	Inactivation	[85]
RhoA, Rac1,Cdc42	YopT, Avr/PhpB	Proteolysis	Inactivation	[61]
RhoA, RhoB, RhoC, Rac1, Cdc42	MARTX	Unknown	Inactivation	[92]
RhoA, Rac1, Cdc42	SopE, SifA, SifB, lpgB1,lpgB2, Map, EspM, EspT	GEF-like activity	Activation	[69]
Arf	RalF	GEF-like activity	Activation	[71]
Rab1	DrrA/SidM	GEF-like activity	Activation	[86–88]
Rab1	DrrA/SidM	GDF-like activity	Activation	[86–88]
RhoA, Rac1, Cdc42	CNF1,CNF2, CNF3	Deamidation†	Activation	[19]
RhoA	CNFY	Deamidation†	Activation	[100]
RhoA, Rac1, Cdc42	DNT	Transglutamination‡	Activation	[19]
Heterotrimeric Gα subunits				
G_i, G_o G_t	PT	ADP ribosylation	Inactivation§	[45,48]
G_q	YpkA	Phosphorylation	Inactivation	[84]
G_s, G_{olf}, G_t	CT, HLT	ADP ribosylation	Activation§	[45,48]
G_q, G_{13}, G_i	PMT	Deamidation	Activation§	[9,21,32,112,113,135]
Large, multidomain GTPases				
EF-2	DT, ExoA	ADP ribosylation	nactivation	[41]

† Deamidation > transglutamination.

‡ Transglutamination > deamidation.

§ Followed by uncoupling of receptor-effector signaling.

CNF: Cytotoxic necrotizing factor; CT: Cholera toxin; DNT: Dermonecrotic toxin; GDF: GDI-displacement factor; GDI: Guanine nucleotide dissociation inhibitor; GET: Guanine nucleotide exchange factor; HLT: Heat-labile enterotoxin; PMT: *Pasteurella multocida* toxin; PT: Pertussis toxin.