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Cellular and Molecular Action of the Mitogenic Protein-Deamidating Toxin from *Pasteurella multocida*

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Summary

The mitogenic toxin from *Pasteurella multocida* (PMT) is a member of the dermonecrotic toxin family, which includes toxins from *Bordetella*, *E. coli* and *Yersinia*. Members of the dermonecrotic toxin family modulate G-protein targets in host cells through selective deamidation and/or transglutamination of a critical active site glutamine residue in the G-protein target, which results in activation of the intrinsic GTPase activity. Structural and biochemical data point to the uniqueness of PMT among these toxins in its structure and action. Whereas the other dermonecrotic toxins act on small Rho GTPases, PMT acts on the α subunits of heterotrimeric G_q, G_i and $G_{12/13}$ protein families. To date, experimental evidence support a model whereby PMT potently stimulates various mitogenic and survival pathways through activation of G_q and $G_{12/13}$ signaling, ultimately leading to cellular proliferation, while strongly inhibiting pathways involved in cellular differentiation through activation of G_i signaling. The resulting cellular outcomes account for the global physiological effects observed during infection with toxinogenic *P. multocida*, as well as hint at potential long-term sequelae that may result from PMT exposure.

Keywords

dermonecrotic toxin; G protein; signal transduction; receptor-mediated endocytosis; deamidation; transglutamination; membrane translocation; cysteine protease; atrophic rhinitis; adipogenesis; mitogenesis; SseI; osteogenesis; pasteurellosis

1. Introduction

Protein toxins have long been known to constitute key virulence determinants for pathogenic bacteria. Recent advances in our understanding of the structural and biochemical basis for the effects of these toxins on various host signaling pathways have provided interesting and sometimes surprising insights into the molecular mechanisms of the pathogenic consequences from exposure to these toxins. Such knowledge has identified toxins as important tools for studying fundamental problems in biology and has also enabled the potential use of these toxins for biomedical applications and as research tools. The emergence of antibiotic resistant, toxin-producing bacteria, along with the heightened awareness of biosecurity threats since 2001, have provided strong impetus to renew our efforts toward understanding toxin-mediated disease processes and discovering alternative antitoxin strategies [1, 2].

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A prominent and prevalent group of bacterial toxins is comprised of large multi-partite proteins (called A-B toxins) that act intracellularly on their targets to modulate host signal transduction and physiological processes. The functional B components (domains or subunits) of A-B toxins bind to host cell receptors and facilitate cellular uptake and delivery of the functional A components into the cytosol, where the A components gain access to and interact with their cellular target or targets to cause toxic effects on the host cell. For a large family of A-B toxins, the intracellular targets are G proteins [3], i.e., GTPases that act as regulatory proteins in eukaryotic cell signaling processes by cycling between an inactive GDP-bound state and an active GTP-bound state. Most A-B toxins modulate their G-protein substrates by locking them, through covalent modification, into either an inactive or an active conformation, thus affecting the downstream signaling pathways.

Members of the dermonecrotic toxin family modulate their G-protein targets through selective deamidation and/or transglutamination of an active site glutamine residue, which results in activation of the intrinsic GTPase activity [3]. The cytotoxic necrotizing factors from *E. coli* (CNF1, CNF2, and CNF3) and *Yersinia* (CNFY) and the dermonecrotic toxin from *Bordetella* spp. (DNT) modify and constitutively activate certain members of the Rho family of small regulatory GTPases, namely RhoA, Rac1 and Cdc42 [4–8] [9–12]. Both CNF1 and CNF2, an presumably CNF3, deamidate a specific Gln residue (Gln-63) of RhoA, as well as Gln-61 of Rac1 and Cdc42 [8, 13–15], while CNFY modifies RhoA but not Rac1 or Cdc42 [16] and DNT activates these proteins primarily through transglutamination of the same Gln residue [15, 17]. This active site Gln residue is located in the switch II region of the G protein and is essential for GTPase activity.

Recently, the potent mitogenic toxin from *Pasteurella multocida* (PMT) joined this group of G-protein-deamidating dermonecrotic toxins, but instead of acting on small Rho GTPases, PMT stimulates various host signal transduction pathways by activating the α subunits of heterotrimeric G proteins of the G_q, G_i and $G_{12/13}$ families (reviewed in [3]). In these Gα proteins, PMT deamidates an active site Gln residue (Gln-209 in $G\alpha_q$, Gln-205 in $G\alpha_i$), which is functionally equivalent to the Gln that is deamidated by the CNFs and DNT [18]. For all of these cases, toxin-catalyzed deamidation or transglutamination of the target inhibits the intrinsic GTPase activity and leads to persistent activation of the regulatory G protein. Although they catalyze the same deamidating reaction on related G-protein targets and with overlapping cellular outcomes, the sequence and structure of the activity domain of PMT differs considerably from that of the other dermonecrotic toxins [3] and points to a clear functional example of convergent toxin evolution. In this review, we will focus on PMT and our current understanding of the structure-function, mechanism of action, and cellular consequences of this newest member of the G-protein-deamidating dermonecrotic toxin family.

2. Epizootic and zoonotic diseases associated with toxinogenic *Pasteurella multocida*

Toxinogenic *P. multocida* is associated with the severest forms of dermonecrosis and pasteurellosis in livestock and other domestic and wild animals [19–22], and is the primary etiological agent of progressive atrophic rhinitis (AR), a disease characterized by destruction of the nasal turbinate bones in pigs, rabbits and other animals [20–26]. While in swine the primary disease manifestation is AR [23], in other animals, such as cattle and rabbits, other symptoms may be more pronounced, including respiratory distress in cattle (bovine respiratory disease) or pneumonia (often referred to as pasteurellosis) in rabbits (snuffles) and abscess formation [20, 27–29]. Systemic effects of toxinogenic *P. multocida* in most animal species include nasal, testicular and splenic atrophy, hepatic necrosis, renal impairment, leukocytosis, symptoms of pneumonia, overall weight loss, growth retardation

and death [20, 23, 28, 30–36]. Toxinogenic *P. multocida* can also affect humans that have contact with infected animals, particularly through respiratory exposure or bite wounds [37–46]. Toxinogenic *P. multocida* is therefore considered a causative agent of both epizootic and zoonotic diseases [37, 47–50].

3. *Pasteurella multocida* toxin and disease

A 1285 amino acid (146-kDa) protein toxin (PMT) associated with serotype D and some A strains of *Pasteurella multocida* is the major virulence factor responsible for bone resorption of nasal turbinates in progressive AR [23, 50], liver necrosis [25, 30, 31, 51], spleen atrophy [23, 31, 52], swelling of the kidneys [25], pneumonia [31], reduced body weight and fat [30, 53], and growth retardation [36, 54, 55]. PMT appears to cause AR through disruption of bone biogenesis and degradation processes, which are mediated by the bone-generating osteoblasts and macrophage-like osteoclasts, respectively [56–58]. In vivo, PMT intoxication stimulates differentiation of preosteoclasts into osteoclasts [59, 60] and promotes osteoclast proliferation, which in turn causes bone resorption [60]. In vitro, PMT stimulates osteoclastic bone resorption [57, 61, 62], while also inhibiting osteoblast differentiation [58, 62–64] and bone regeneration by osteoblasts [57, 58, 64].

4. Cellular activity of PMT

Intoxication of mammalian cells by PMT induces strong mitogenic [65–67] and anti-apoptotic effects [68–70] in various cell lines. The cellular effects of PMT are induced by the activation of heterotrimeric G proteins of at least three different families (G_q, G_i, and $G_{12/13}$), which lead to mitogenic responses through increased intracellular Ca^{2+} and inositol phosphate levels due to activation of phospholipase Cβ (PLCβ) [71, 72] and activation of Rho-dependent cytoskeletal signaling [73–75], while concurrently shutting off cAMP-dependent signaling pathways leading to differentiation [68, 76].

Some of the intracellular events that occur upon exposure to PMT are: enhanced hydrolysis of inositolphospholipids to increase the total intracellular content of inositol phosphates [71, 72] increased production of diacylglycerol [77], mobilization of intracellular Ca^{2+} pools [68, 71, 72, 78]; interconversion of GRP78/BiP [79]; and activation of protein kinase C-dependent and -independent phosphorylation [66, 67, 70, 77, 80–82]. Activation of these pathways leads to subsequent alteration of downstream gene expression by activation of Ca^{2+} [68, 78, 83], MAPK [66, 67, 83, 84] and JAK/STAT [85–87] signaling pathways and inhibition of G_s-mediated signaling pathways. A summary of the various intracellular signal transduction pathways affected by PMT treatment is shown in Figure 1. We will explore in turn the action of PMT on each of these signaling pathways.

Calcium Signaling

Exposure of cultured fibroblasts and osteoblasts to PMT results in the activation of phosphatidylinositol-specific phospholipase C (PLCβ) [58, 71, 77], which in turn triggers hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to increase intracellular levels of inositol 1,3,5-trisphosphate (IP_3) and diacylglycerol (DAG) and stimulates downstream Ca^{2+} signaling pathways. PMT strongly stimulates primarily PLCβ1 and to a lesser extent PLCβ3, but not PLCβ2 [72]. These findings are consistent with known cellular PLCβ responses elicited through $G\alpha_q$-coupled receptors [88], and indeed, activation of PLCβ1 occurs through selective action of PMT on the regulatory $G\alpha_q$ subunit, but not the closely related $G\alpha_{11}$ subunit [72, 84]. Discrimination between $G\alpha_{11}$- and $G\alpha_q$-mediated activation of PLCβ by PMT was attributed to the helical domain of the heterotrimeric G proteins [89], although it is not clear whether the basis for this discrimination occurs as a result of

differential recognition of the $G\alpha_q$ versus $G\alpha_{11}$ protein by PMT or through preferential coupling of the $G\alpha_q$ versus $G\alpha_{11}$ protein to the downstream PLCβ effector protein.

The PMT-induced PLCβ response was potentiated by release of the $G\alpha_q$ subunit from the heterotrimeric $G\alpha\beta\gamma$ complex through either dissociation of the $G\alpha_q$ subunit from $G\beta\gamma$ by using antibodies against the Gβ subunit or through sequestration of the $G\beta\gamma$ subunits away from the $G\alpha_q\beta\gamma$ heterotrimeric complex by treatment with pertussis toxin (PT) [72]. PMT action on $G\alpha_q$ is irreversible and persistent [72, 90] and independent of interaction with G protein-coupled receptors [90]. Indeed, PMT potentiates the PLCβ response elicited by $G\alpha_q$-coupled receptors upon stimulation with bombesin, vasopressin or endothelin [91]. Furthermore, overexpression of $G\alpha_q$ enhanced the PMT-induced response, while decreased expression of $G\alpha_q$ or treatment with the GDP analogue, GDPβS, which locks G proteins in their inactive form, blocked the PMT-induced response [72], supporting the monomeric form of $G\alpha_q$ as the preferred substrate of PMT. However, after the strong initial PMT-induced response an uncoupling of the $G\alpha_q$-coupled PLCβ signaling pathway subsequently follows, such that no further stimulation occurs upon additional treatment with PMT [72].

Release of the second messengers IP_3 and DAG mediated by PMT leads to stimulation of Ca^{2+} signaling through mobilization of intracellular Ca^{2+} stores [71, 72, 78, 84] and activation of Ca^{2+}-dependent protein kinase (PKC)-catalyzed phosphorylations [70, 77], Ca^{2+}-calmodulin (CaM)-calcineurin-dependent nuclear factor of activated T cells (NFAT) signaling [68], and Ca^{2+}-dependent Cl^- secretion [72, 92].

Mitogenic Signaling

PMT exhibits proliferative or cytopathic effects on a number of cultured cell lines. In cultured mesenchymal cells, such as murine, rat, and human fibroblasts [65–67], preadipocytes [68], and osteoblasts [57, 58], PMT elicits primarily a proliferative response, leading to the speculation that PMT can promote cancer [87, 93]. Accordingly, PMT initiates intracellular signal transduction events that result in DNA synthesis and cytoskeletal rearrangements. In agreement with these findings, PMT stimulates fibroblastic cells through the cell cycle, moving cells from the G1 phase into and through the S phase without triggering apoptosis [67]. Consistent with these observations, PMT treatment induces the expression of a number of cell cycle markers, including the protooncogene c-Myc, D and E cyclins, PCNA, p21, and the Rb proteins. Yet, continued expression of these markers is not sustained after the initial proliferative response and confluent Swiss 3T3 cells become unresponsive to further PMT treatment [67].

In contrast, PMT causes cytopathic responses in other cell types, such as cultured epithelial cells, including embryonic bovine lung (EBL) cells [94], Vero cells [67, 95, 96], cardiomyocytes [70], and osteosarcoma cells [96]. For example, confluent Vero cells undergo rapid and dramatic morphological changes upon toxin exposure [67, 95, 96]. But, PCNA and cyclins D3 and E are not upregulated in these cells upon PMT treatment, and so no cell cycle progression occurs; instead, cells arrest primarily in G1 [67].

Mitogenic signaling stimulated by PMT appears to be different for different cell types. For example, in HEK-293 cells PMT induces Ras-dependent activation of Erk mitogen-activated protein kinase (MAPK) via Gq-dependent, but PKC-independent transactivation of the epidermal growth factor (EGF) receptor [66], which was blocked by cellular expression of two inhibitors of G_q signaling, a dominant-negative mutant of the G-protein-coupled receptor kinase 2 (GRK2) and a C-terminal peptide of $G\alpha_q$ (residues 305–359). Consistent with this, Erk activation by PMT was insensitive to the PKC inhibitor (GF109203X), but was blocked by tyrphostin (AG1478), an EGF receptor-specific inhibitor, and by dominant

negative mutants of mSos1 and Ha-Ras. In cardiac fibroblasts Erk activation by PMT also occurs via transactivation of the EGF receptor, resulting in fibrosis [70].

In cardiomyocytes, however, PMT-induced activation of Erk and to a lesser extent JNK and p38 MAPK occurs via G_q-dependent activation of PLCβ and novel PKC (nPKC) isoforms [70], resulting in cardiomyocyte hypertrophy reminescent of that induced by norepinephrine activation of G_q-coupled receptors [97]. Similar to norepinephrine, PMT suppresses activation of Akt, a serine/threonine protein kinase that is activated by Gβγ subunits and Ras GTPases, and causes apoptosis, albeit not to the extent of norepinephrine [70]. PMT also induces serine phosphorylation of p66Shc, an adapter protein of oxidative stress responses, via PKC and MEK signaling [81], suggesting that p66Shc might be a candidate mediator of PMT-enhanced apoptosis in cardiomyocytes.

On the other hand, PMT also activates anti-apoptotic pathways. For example, PMT activates protein kinase D (PKD) signaling in both cardiac fibroblasts and cardiomyocytes [82], presumably through DAG-dependent phosphorylation by nPKC [98], which leads to phosphorylation of the transcription factor cAMP response element-binding protein (CREB) and increased expression of CREB target genes such as the anti-apoptotic Bcl-2 protein.

Additional evidence pointing to the oncogenic potential of PMT is the finding that PMT treatment leads to activation of JAK/STAT signaling [86, 87]. Treatment of Swiss 3T3 cells with PMT results in G_q-dependent phosphorylation and activation of the Janus tyrosine protein kinases, JAK1 and JAK2 [87]. This is followed by JAK-mediated activation of STAT1, STAT3 and STAT5 through tyrosine phosphorylation, and at least in the case of STAT3 further activation through subsequent serine phosphorylation. PMT-stimulation of phosphorylation of STAT transcription factors leads to upregulation of cyclooxygenase 2 (Cox-2), a pro-inflammatory protein upregulated in many cancers, but downregulation of the transcription factor suppressor of cytokine signaling 3 (SOCS-3) [87]. In HEK-293 cells PMT also increases expression of the serine/threonine protein kinase Pim-1, which phosphorylates and inactivates the transcription factor SOCS-1 [86]. Phosphorylated SOCS-1 can no longer act as an E3 ubiquitin ligase to target JAK proteins for proteosomal degradation, thereby leading to increased levels of JAK.

Cytoskeletal Signaling

PMT initiates cytoskeletal rearrangements, including focal adhesion assembly and actin stress fiber development [11, 80, 99, 100]. These actin cytoskeletal rearrangements appear to be RhoA-dependent [11, 67, 73, 75, 80, 101]; however, PMT does not act directly on RhoA [6, 11, 102]. Instead, RhoA activation occurs through PMT activation of $G\alpha_{12/13}$ [74], presumably by interaction of the Gα subunit with the RhoGEFs p115-RhoGEF, PDZ-RhoGEF, LARG, or Dbl [103–106]. RhoA activation indirectly through PMT activation of $G\alpha_q$ [72, 84], presumably by interaction of the Gα subunit with the regulator of G protein signaling (RGS) domain of the Rho guanine nucleotide exchange factor (RhoGEF), p63RhoGEF [107] or Lbc [75]; or. In $G\alpha_{q/11}$-deficient fibroblasts, expression of dominant-negative $G\alpha_{13}$ inhibited RhoA activation by PMT, whereas in $G\alpha_{12/13}$-deficient cells expression of $G\alpha_{13}$ restored RhoA activation by PMT [74]. Whether PMT can discriminate between the $G\alpha_{12}$ and $G\alpha_{13}$ proteins remains to be determined.

PMT-induced RhoA activation subsequently leads to activation of its downstream target Rho kinase (ROKα), which then phosphorylates and inactivates myosin light chain (MLC) phosphatase PP1 and thereby leads to increased levels of MLC phosphorylation [101]. The resulting MLC phosphorylation regulates actin reorganization, increasing stress fiber formation, cell retraction and endothelial cell layer permeability [101]. PMT-mediated RhoA activation also promotes to ROKα-dependent autophosphorylation of focal adhesion

kinase (FAK) on Tyr-397, which is an SH2-binding site for Src tyrosine kinase [80, 100]. This binding results in formation of a FAK-Src complex that leads to further tyrosine phosphorylation of downstream adaptor proteins such as paxillin and Cas and facilitates stress fiber formation and focal adhesion assembly [80, 100].

PMT-mediated RhoA activation and subsequent disturbance of endothelial barrier function has been speculated to be responsible for the vascular effects of PMT observed in dermonecrotic lesions from bite wounds [73]. This is consistent with histological observations showing evidence of endothelial damage by influx of neutrophils and increased attachment of thrombocytes to blood vessels surrounding PMT-induced dermal lesions [108].

cAMP signaling

In addition to activation of $G\alpha_q$ and $G\alpha_{12/13}$ signaling, PMT treatment inhibits adenylyl cyclase activity through activation of $G\alpha_i$ [76], converting it into a PT-insensitive state. Capitalizing on the fact that the preferred substrate of PT-catalyzed ADP-ribosylation is the heterotrimeric $G\alpha_i\beta\gamma$ complex, and not the monomeric $G\alpha_i$ [109], it was found that PMT action on the $G\alpha_i$ protein interferes with the interaction of $G\alpha_i$ and its cognate $G\beta\gamma$ subunits and thereby prevents ADP-ribosylation by PT [76], resulting in activation and subsequent uncoupling of $G\alpha_i$ signaling. In this study, PMT treatment of intact wild-type mouse embryonic fibroblasts, as well as cells deficient in $G\alpha_{q/11}$ or $G\alpha_{12/13}$, resulted in inhibition of cAMP accumulation through isoproterenol stimulation of G_s-coupled receptors or through forskolin stimulation of adenylate cyclase activity, while enhancing the inhibition of cAMP accumulation by lysophosphatidic acid (LPA) through G_i-coupled receptors. While PT treatment blocked LPA-mediated inhibition of cAMP accumulation, it did not block PMT-mediated activation of $G\alpha_i$ or inhibition of cAMP accumulation. The observation that PMT overrides the action of PT suggests that PMT may also be able to act on the heterotrimeric $G\alpha_i\beta\gamma$ complex.

The effect of PMT on the GTPase activity of the $G\alpha_i$ subunit has also been studied. PMT treatment of cells not only reduced both basal and LPA-induced hydrolysis of GTP by the $G\alpha_i$ protein in membrane preparations, but also inhibited LPA-receptor-stimulated binding of $GTP\gamma S$ to $G\alpha_i$ [76], suggesting that PMT locks the $G\alpha_i$ subunit in its monomeric active form. The finding that pretreatment of cells with PMT prevented PT-induced ADP-ribosylation of $G\alpha_{i2}$ is in keeping with the proposed model where PMT acts on the $G\alpha$ subunit to irreversibly convert it into an active state that can no longer interact with its cognate $G\beta\gamma$ subunits [72]. This effectively shifts the equilibrium to dissociate the heterotrimeric complex and release the $G\beta\gamma$ subunits, which can then interact with their downstream effector proteins, such as phosphoinositide 3-kinase γ (PI3Kγ). Activation of PI3Kγ generates PIP_3 that activates phosphoinositide-dependent protein kinase 1 (PDK1), which then phosphorylates Akt and upregulates Pim-1 expression, thereby stimulating survival pathways while inhibiting apoptotic pathways [69].

Adipogenesis

PMT prevents adipocyte differentiation and blocks adipogenesis [68]. After hormonal stimulation with a combination of insulin, dexamethasone and isobutylmethylxanthine, confluent 3T3-L1 fibroblastic preadipocyte cells are induced to differentiate by first entering a mitotic clonal expansion stage with increased expression of cell cycle markers such as cyclins and c-Myc, which is then followed by subsequent growth arrest and terminal differentiation into mature adipocytes containing abundant lipid droplets [110], which are visualized by Oil Red O staining. PMT completely blocks this process in 3T3-L1 cells [68].

During adipocyte differentiation Notch1 signaling plays a pivotal role in regulating expression of adipocyte-specific markers [111]. The transcription factors peroxisomal proliferator-activated receptor (PPARγ) and CAATT enhancer-binding protein (C/EBPα), are upregulated [110], while preadipocyte-specific markers, such as Pref1 [112], and Wnt/β-catenin signaling [113] are downregulated. PMT prevents expression of PPARγ and C/EBPα in 3T3-L1 preadipocytes and downregulated expression of PPARγ and C/EBPα in mature adipocytes [68]. PMT completely downregulates Notch1 levels, yet maintains high levels of Pref1 and β-catenin [68].

Although the connection between G_q-dependent Ca^{2+} signaling and Notch1 signaling in adipogenesis is not fully understood, G_q-mediated Ca^{2+} signaling blocks adipogenesis through activation of the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin [114, 115]. However, the inhibitory effects of PMT on differentiation and Notch1 could not be reversed by treatment with the calcineurin inhibitor cyclosporin A, suggesting that PMT-mediated blockade of adipocyte differentiation must occur through multiple pathways. PMT activation of G_i signaling, which would block G_s-mediated differentiation, might account for these inhibitory effects. These results regarding PMT action on adipogenesis may account in part for the decreased weight gain and growth retardation observed in animals exposed to PMT [30, 36, 53–55, 96].

Osteogenesis

Natural or experimental exposure to PMT in animals causes bone loss in nasal turbinates [116, 117], calvaria [61] and long bones [60]. In tissue culture, PMT stimulates proliferation of primary mouse calvaria and bone marrow cells [57, 58, 60], while inhibiting differentiation of osteoblasts to bone nodules through activation of the RhoA-ROKα signaling pathway [63]. PMT downregulates the expression of several markers of osteoblast differentiation, including alkaline phosphatase and type I collagen [57]. Overall bone loss mediated by osteoclasts appears to require interaction of PMT-stimulated osteoblasts [58], presumably through cytokines released by activation of the osteoblasts [62]. While PMT appears to stimulate preosteoclasts (bone marrow progenitor cells) to differentiate into osteoclasts [59], it was recently shown that PMT-induced osteoclastogenesis is mediated indirectly through a subset of B cells that are activated by PMT to produce osteoclastogenic factors and cytokines [85].

The Notch1 signaling pathway also plays an important role in regulation of osteogenesis by blocking osteoblastic cell differentiation [111, 118]. The observation that PMT downregulates Notch1, while maintaining β-catenin levels to blocking adipogenesis [68], suggests that these signaling pathways may also play a role in PMT-induced bone resorption.

Immune Signaling

Even though immunization with PMT toxoid affords protection [119–124], naturally occurring AR is characterized by an overall lack of immune response against PMT [125–128]. Immunization with killed toxigenic *P. multocida* bacteria generated only low levels of toxin-neutralizing antibodies [53, 120, 129]. Although PMT activates dendritic cells [125, 130], it is a poor immunogen and appears to suppress the antibody response in vivo [125–127] and inhibits immune cell differentiation and dendritic cell migration [63, 125, 130]. Vaccination with PMT showed lower IgG antibody responses against other antigens, including limpet hemocyanin, ovalbumin, and tetanus toxoid [126, 127], suggesting a possible role for PMT as an immunomodulator in pathogenesis.

5. PMT structure and enzyme activity

Dermonecrotic toxin family

One question of interest is the relationship between the structural similarities and the activities of PMT with the related DNT and CNFs. All three toxins cause similar, but not identical, effects on cultured cells [6, 11, 80, 99, 102]. Although there is no crystal structure for any of the full-length proteins, sequence comparisons and biochemical studies provide insights into the functional organization of these toxins. Although the precise localization of the domains responsible for receptor binding and translocation activities remains unclear, these domains are located in the N terminus of each of these toxins and share limited sequence similarities with each other [131–137] and will be discussed in more detail later in section 6. On the other hand, more is known about the intracellular activity domain, which resides in the C terminus of each toxin [14, 83, 132, 133, 138, 139]. Crystal structures of C-terminal fragments of PMT (PDB 2EBF) [139] and CNF1 (PDB 1HQ0) [138] are available and have revealed that they are quite different from each other.

The deamidase activity of CNF1 involves two essential C-terminal Cys and His residues [140], which are conserved in all members of the CNF/DNT family (Cys-866 and His-881 in CNF1, Cys-1305 and His-1320 in DNT). Since DNT and the CNFs share sequence similarity in their C-terminal domains (residues 720–1014 in the CNFs, 1176–1464 in DNT) and have common G protein targets, it is presumed that their activity domains have similar overall structures. PMT does not share any discernable sequence similarity with the C-terminal regions of DNT or the CNFs and the solved crystal structure of a biologically active C-terminal fragment of PMT (PMT-C) consisting of residues 575–1285 also showed no structural similarity [139]. The structure of PMT-C revealed three distinct domains: a C1 domain (residues 575–719) with sequence and structural similarity to the membrane-targeting domain of the clostridial toxin TcdB [141, 142]; a C2 domain (residues 720–1104) of as-yet unknown function; and a C3 domain (residues 1105–1285) with a papain-like cysteine protease structural fold that was subsequently shown to harbor the minimal domain responsible for toxin-mediated activation of Ca^{2+} and mitogenic signaling [83].

Comparison of PMT-C3 with TGase (Pf01841) and NAT (Pf00797) families

PMT-C3 has structural similarity with the catalytic core of transglutaminases (TGase family Pf01841) and arylamine N-acetyltransferases (NAT family Pf00797) [18]. The spatial arrangement of the active site Cys-His-Asp triad of PMT-C3 is nearly superimposable with members of the TGase and NAT families [3], including the human blood-clotting factor XIII (PDB 1FIE) [143], fish-derived TGase from red sea bream (PDB 1G0D) [144], putative TGase-like cysteine protease from *Cytophaga hutchinsonnii* (PDB 3ISR), and the arylamine N-acetyltransferase (NAT) from *Salmonella enterica* serovar Typhimurium (PDB 1E2T) [145]. The structure of PMT-C3 most closely resembles that of the protein glutaminase from *Chryseobacterium proteolyticum* (PDB 2ZK9) [146], which shares some weak sequence similarity with PMT-C3 and also has a Cys-His-Asp triad superimposable with this group of proteins, but does not belong to either of the TGase or NAT families. The crystal structures of another family of bacterial type 3 secretion system (T3SS) effector proteins, called CIF (cycle inhibiting factor) from *E. coli* (PDB 3EFY)[147] and CIF homologs from *Burkholderia pseudomallai* (CHBP, PDB 3EIT) [148, 149], *Photorhabdus luminescens* (PDB 3GQJ) [149], and *Yersinia* species [149], have revealed active site Cys-His-Gln/Asp motifs associated with CIF-mediated actin stress fiber formation and cell cycle arrest [150, 151]. Recently, CIF and CHBP were shown to selectively deamidate Gln-40 in ubiquitin and the ubiquitin-like protein NEDDS, thereby blocking the ubiquitination-proteosome pathway [152].

Other PMT-C3-related bacterial proteins

A striking finding about the group of proteins with Cys-His-Asp triads similar to that of PMT-C3 is that at the sequence level there is no discernable similarity of PMT-C3 to the proteins, with the exception of the *Cryseobacterium* protein glutaminase. On the other hand, there are a group of proteins with activity domains that have recognizable sequence similarity to PMT-C3 (Figure 2), even though there is no structural confirmation of this yet. Most notable are several related SPI-2 T3SS effector proteins from *Salmonella enterica* serovars and *Arsenophonus nasoniae*, an insecticidal toxin from *Photorhabdus asymbiotica*, and a number of hypothetical bacterial proteins from *Vibrio coralliilyticus, Vibrio fischeri, Erwinia tasmaniensis, Mesorhizobium* sp., *Chromobacterium violaceum,* and *Yersinia mollaretii*. Among these are the recently characterized T3SS effector proteins SseI (also called SrfH) from *S. enterica* serovar Typhimurium [153] and its close homologs. SseI binds to and inhibits the host factor, IQ motif-containing GTPase-activating protein 1 (IQGAP1), which in turn inhibits cytoskeletal signaling and migration of macrophages and dendritic cells, thereby preventing bacterial clearance during infection [153]. Each of these proteins share the highly conserved active site Cys-His-Asp triad found in PMT-C3, as well as additional conserved Trp and Gln-Phe residues (highlighted in Figure 2). Mutation of the active site Cys-178 to Ala in SseI results in loss of function, but not binding to IQGAP1 [153].

Substrate specificity of PMT

The active site Gln residue located in the switch II region of GTPases serves to stabilize the pentavalent transition state for GTP hydrolysis and serves to orient the water nucleophile. Deamidation of this Gln in $G\alpha_i$ or $G\alpha_q$ by PMT constitutively activates and releases the Gα subunit from the respective heterotrimeric Gαβγ complex [18]. So far, detection of PMT-catalyzed deamidase activity of Gα proteins in vitro has proven to be a challenge, and most biochemical studies to date have relied on whole cell studies to address questions regarding substrate specificity and effects of PMT action on Gα protein interactions with its cognate Gβγ subunits, receptors, effectors, and/or regulators.

One enigmatic aspect of PMT action on its G protein substrates is the ability of PMT to discriminate among the different Gα isoforms since all of the Gα subunits have analogous active site Gln residues (equivalent to Gln-204 of $G\alpha_{i1}$, Gln-205 of $G\alpha_{i2}$, Gln-229 of $G\alpha_{12/13}$, and Glu-209 of $G\alpha_{q/11}$) and share significant sequence similarity in the flanking sequences of the switch II region (see Figure 3). It is noteworthy that $G\alpha_q$ and $G\alpha_{11}$ share considerable amino acid identity (88% overall) with each other, including the switch II Gln-209 residue, yet only $G\alpha_q$ is a substrate for PMT [18, 84]. The reason for this difference in substrate specificity between $G\alpha_q$ and $G\alpha_{11}$ is not known; however, there is some evidence that differences in substrate recognition of $G\alpha_q$ versus $G\alpha_{11}$ by PMT may reside in the helical domain of the Gαprotein [89].

Although direct deamidation of $G\alpha_{12}$ or $G\alpha_{13}$ by PMT has not yet been demonstrated, exogenous expression of $G\alpha_{13}$ restored RhoA activation by PMT in $G\alpha_{12/13}$-deficient cells in the presence of the specific $G\alpha_q$ inhibitor YM-254890 [74], indicating that $G\alpha_{13}$ can serve as a substrate for PMT. PMT-mediated activation of $G\alpha_{12}$ signaling was not tested in this study, but since both $G\alpha_{12}$ and $G\alpha_{13}$ share 67% overall amino acid identity and nearly identical switch II regions (Figure 3), it is possible that they could both serve as a target for PMT-mediated deamidation. However, considering that $G\alpha_q$ and $G\alpha_{11}$ share even greater similarity, yet $G\alpha_{11}$ is not a substrate of PMT, it will be interesting to see whether $G\alpha_{12}$ is a substrate for PMT. It also remains to be determined which of the other G protein α subunits might also be targets for deamidation by PMT.

6. Cellular intoxication of PMT

Little is known about the cellular uptake mechanisms of PMT. Bacterial protein toxins are known to utilize a number of different entry routes. And, the nature of the receptor often dictates what route is taken. It has been suggested that the cellular receptor for PMT might be a ganglioside [96, 99], but the identity of the receptor(s) responsible for PMT binding to host cells remains unclear. Once PMT binds to cells, it is internalized through a receptor-mediated endocytic pathway involving a pH-dependent step [62, 65, 96]. Although the detailed mechanism of this process is lacking, the toxin is translocated from the endocytic vesicles into the cytosol, where the C-terminal activity domain gains access to its target to activate intracellular signaling.

Weak bases such as NH_4Cl, chloroquine, and methylamine, which buffer the acidification of endosomes, block PMT activity on cells [65]. Bafilomycin A1, a potent and specific inhibitor of the vacuolar H^+-ATPase responsible for endosomal acidification, was also found to inhibit PMT activity [154, 155]. Involvement of a low pH-dependent membrane translocation event in PMT action was further supported by entry of cell surface-bound PMT directly into cells by a low pH pulse at 4°C in the presence of bafilomycin A1 [154]. There is some evidence that a predicted helix-loop-helix motif, entailing two hydrophobic helices (residues 402–423 and 437–457) linked by a hydrophilic loop (residues 424–436), may be part of a pH-sensitive membrane translocation domain of PMT [154]. Double mutation of Asp-373 and Asp-379 in the corresponding helix-loop-helix of CNF1 resulted in complete loss of biological activity [136]. Substitution of putative pH-sensitive acidic residues in the loop (Asp-425, Asp-431, Glu-434) of PMT with Lys resulted in reduction in toxin activity, whereas mutation of Asp-401 in PMT, which is outside of the helix-loop-helix motif, completely abolished PMT activity [154].

A recent study showed that endocytosis and trafficking of PMT is dependent on the small regulatory GTPase Arf6 [155]. In this study, PMT was found to share an initial entry pathway through Arf6-containing endosomes with transferrin and cholera toxin. However, the trafficking pathways subsequently diverge, as transferrin is trafficked to recycling endosomes, cholera toxin is trafficked retrograde to the ER, and PMT is trafficked to late endosomes. Nocodazole, which causes disassembly of microtubules important for trafficking from early to late endosomes, and cytochalasin D, which disrupts actin polymerization, blocked PMT activity [155], suggesting that membrane translocation and cytotoxicity of PMT is dependent on its trafficking to late acidic endosomes. Interestingly, disruption of Golgi-ER trafficking with brefeldin A increased PMT activity, suggesting that inhibiting PMT trafficking to non-productive compartments that do not lead to translocation, enhances PMT translocation and activity.

7. Perspective

A large number of bacterial toxins act on host cell G proteins that regulate a myriad of cellular signaling pathways. Many do so through covalent modification of their targets, such as ADP-ribosylation, monoglucosylation, phosphorylation, proteolysis, deamidation or transglutamination, while others do so through noncovalent interactions involving GEF-like, GAP-like, GDI-like or GDF-like activities [3]. In the case of signaling by heterotrimeric G-protein-coupled receptors, CT directly activates G_s and PT directly inhibits G_i and G_o, thereby indirectly potentiating G_s signaling, while PMT directly activates G_i, thereby indirectly inhibiting G_s signaling. PMT also directly activates a number of other signaling pathways, including G_q, $G_{12/13}$, and possibly others that have not been delineated yet. CT and PT modify their targets through ADP-ribosylation, but PMT does so by deamidation.

The realization that a number of bacteria, including known pathogens such as *Pasteurella*, *Bordetella*, *Yersinia*, *Salmonella*, *Burkholderia* and *E. coli*, produce deamidases/TGases that affect cytoskeletal and mitogenic signaling, suggests that there may be additional bacteria that produce such factors to modulate host signaling during infection. Modification of heterotrimeric G proteins through deamidation so far is unique to PMT. The finding that SseI may also be a deamidase, sharing partial amino acid sequence similarity with PMT in addition to the same catalytic triad, supports this notion. Availability of current genomic sequences and structural databases may enable further discoveries through gene and protein sequence comparisons, particularly with use of the Cys-His-Asp triad motif to point toward potential candidates. One difficulty encountered in finding more potential deamidase/TGase-like bacterial factors is detection of the modification, which involves detection of a molecular mass change of 1 dalton in a relatively large protein substrate (20–45 kDa). Another challenge is development of an in vitro assay for studying the enzyme activity of these proteins.

Although PMT has multiple intracellular G protein targets, the overall outcome (summarized in Figure 1) is downregulation of differentiation, such as adipogenesis and osteogenesis, with concomitant upregulation of mitogenesis and cell growth, without inducing apoptosis. The cellular actions of several bacterial toxins, including PMT, have been linked to their effects on mitogenic signaling, and have been speculated as potential contributors to cancer predisposition as long-term sequelae to bacterial infection [87, 93, 156]. Further exploration of toxin action may provide additional insights into the regulation of cellular processes and cell fate decisions.

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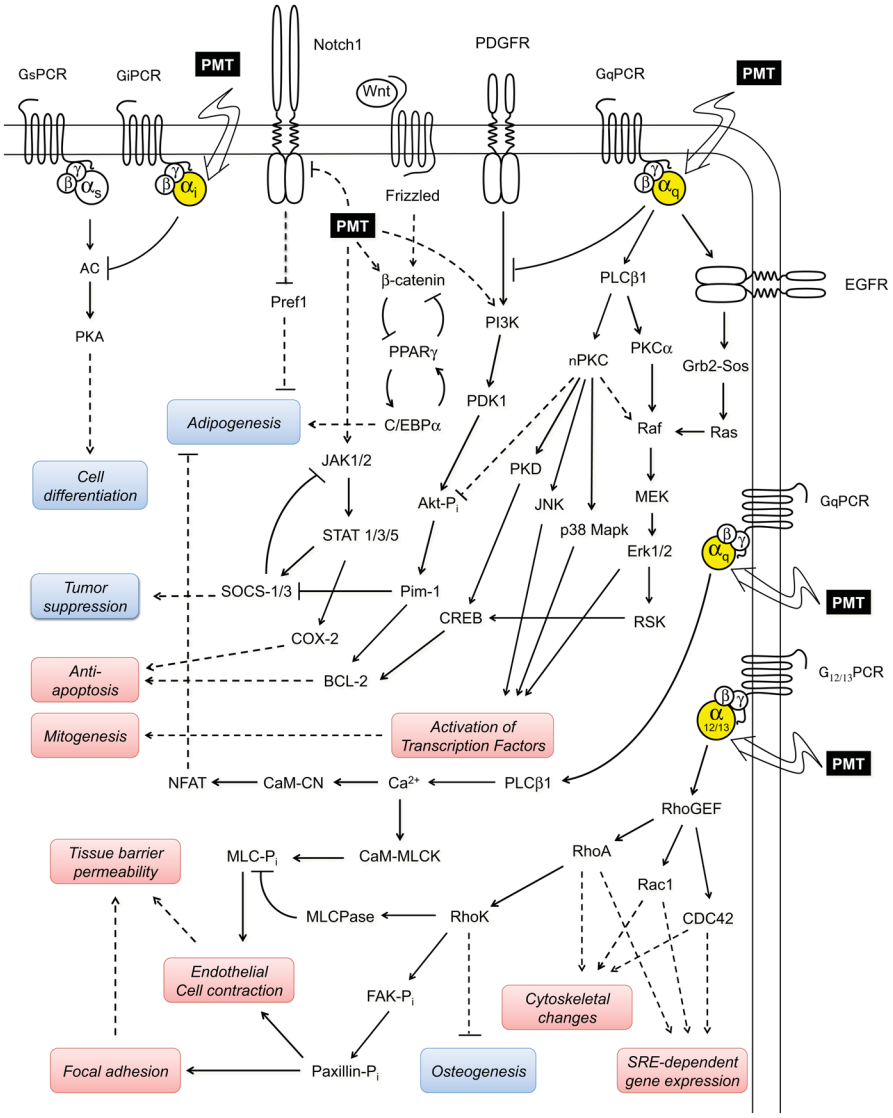

Figure 1. Known intracellular signaling pathways involved in PMT action on host cells

Overall cellular outcomes that are enhanced by PMT are indicated in red boxes, while outcomes that are blocked by PMT are indicated in blue boxes. Known direct target substrates of PMT ($G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$) are indicated in yellow. Arrows point in the direction of positive/activation of the signaling pathway, while barred lines indicate negative/inhibition of the signaling pathway. Solid lines indicate interactions that are known to be direct, while dashed lines indicate indirect interactions or effects. P_i indicates phosphorylation of the signaling molecule. Abbreviations: GsPCR, G_s-protein coupled receptor; G_iPCR, G_i-protein coupled receptor; G_qPCR, G_q-protein coupled receptor; $G_{12/13}$PCR, $G_{12/13}$-protein coupled receptor; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; PPAR, peroxisome-proliferator activated receptor; GIRK, G-protein-coupled inward-rectifying K^+ channel; AC, adenylate cyclase; PKA, cAMP-dependent protein serine/threonine kinase A; PKD, DAG-dependent serine/threonine protein kinase D; C/EBP, CAATT-enhancer binding protein; Pref1, preadipocyte factor 1; JAK, Janus tyrosine protein kinase; STAT, signal transducer and activator of transcription; SOCS, suppressors of cytokine signaling; Cox-2, cyclooxygenase-2; Pim-1,

Pim serine/threonine protein kinase-1; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide-dependent protein kinase 1; Akt, (also PKB) serine/threonine protein kinase; Frizzled, Wnt-activated G-protein coupled receptor; β-catenin, subunit of the cadherin adherens junction protein complex; Bcl-2, B-cell lymphoma 2 anti-apoptotic protein; NFAT, nuclear factor of activated T cells transcription factor; CN, calcium-calmodulin-dependent calcineurin protein phosphatase; CaM, calcium-dependent calmodulin; MLCK, myosin light chain kinase (serine/threonine protein kinase); MLCPase, myosin light chain phosphatase; Ras, Ras small regulatory GTPase; RhoA, RhoA small regulatory GTPase; Rac, Rac1 small regulatory GTPase; CDC42, Cdc42 small regulatory GTPase; RhoK, Rho kinase ROKα; FAK, p125FAK focal adhesion tyrosine protein kinase; paxillin, focal adhesion adaptor protein; PLCβ, phosphatidylinositol-dependent phospholipase C β isoform; PKC, calcium-dependent serine/threonine protein kinase C; nPCK, novel PKC; Raf, Ras-activated factor serine/threonine protein kinase; Grb2, growth factor receptor-bound adaptor protein 2; Sos, son of sevenless guanine nucleotide exchange factor for Ras; MAPK, mitogen-activated protein serine/threonine protein kinase; MEK, MAPK serine/threonine protein kinase; Erk1/2, (also p42/p44 MAPK) extracellular signal-regulated serine/threonine protein kinase; JNK, (also MAPK10) c-Jun N-terminal serine/threonine protein kinase; RSK, ribosomal S6 serine/threonine protein kinase; CREB, cAMP responsive element binding protein 1 transcription factor.

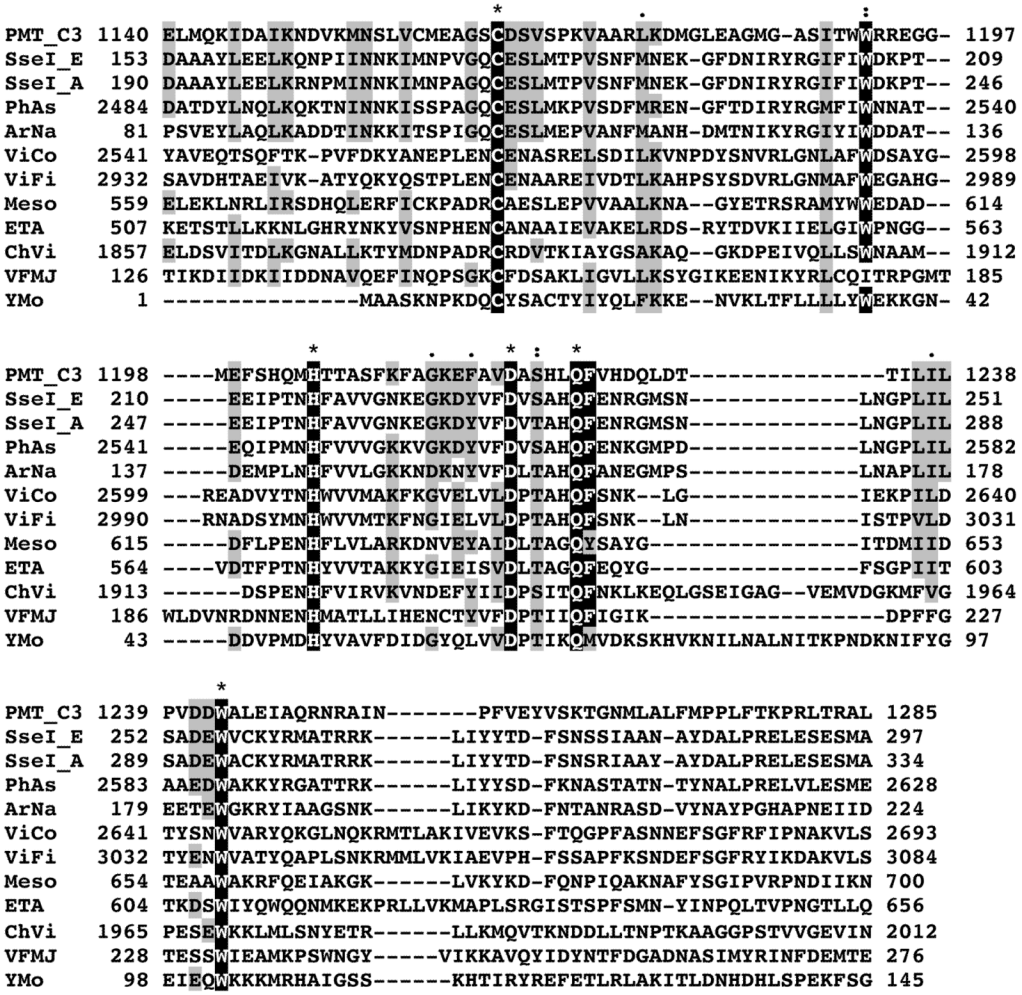

Figure 2. Alignment of amino acid sequences with similarity to PMT-C3

The protein sequences were obtained from NCBI; **PMT_C3:** C3 domain of *Pasteurella multocida* toxin; **SseI_E:** SseI from *Salmonella enterica* serovar Enteritidis; **SseI_A:** SseI from *Salmonella enterica* serovar Arizonae; **PhAs:** insecticidal toxin from *Photorhabdus asymbiotica*; **ArNa:** secreted effector protein from *Arsenophonus nasoniae*; **ViCo:** hypothetical protein VIC_001387 from *Vibrio coralliilyticus*; **ViFi:** hypothetical protein VF_A1129 from *Vibrio fischeri* strain ES114; **Meso:** hypothetical protein Meso_3517 from *Mesorhizobium* sp. Strain BNC1; **ETA:** hypothetical protein ETA_29930 from *Erwinia tasmaniensis* strain Et1/99; **ChVi:** hypothetical protein CV_2593 from *Chromobacterium violaceum*; **VFMJ:** hypothetical protein VFMJ11_A0013 from *Vibrio fischeri* strain MJ11; **YMo:** hypothetical protein ymoll0001_35050 from *Yersinia mollaretii*. The numbers at the ends of each line correspond to the amino acid position in the indicated protein. The catalytic Cys-His-Asp triad as well as the highly conserved Trp and Gln-Phe residues are highlighted in black, “*” denotes identical amino acid residues; “:” denotes highly conserved residues; “.” denotes conserved residues.

Figure 3. Alignment of amino acid sequences of the switch II region in the α subunits of heterotrimeric GTPases

The protein sequences were obtained from NCBI; $G\alpha_{t1}$ (NP_032166), $G\alpha_{t2}$ (NP_032167), $G\alpha_{i1}$ (NP_034435), $G\alpha_{i2}$ (AAH65159), $G\alpha_{i3}$ (NP_034436), $G\alpha_{oA}$ (NP_034438), $G\alpha_{oB}$ (P18873.3), $G\alpha_{z}$ (NP_034441), $G\alpha_{s}$ (P63094), $G\alpha_{olf1}$ (NP_034437), $G\alpha_{olf2}$ (NP_796111), $G\alpha_{11}$ (NP_034431), $G\alpha_{q}$ (NP_032165), $G\alpha_{14}$ (NP_032163), $G\alpha_{15}$ (NP_034434), $G\alpha_{12}$ (NP_034432), and $G\alpha_{13}$ (NP_034433). The numbers below the alignment correspond to the amino acid positions of $G\alpha_{q}$. The active site Gln (at position 209 in $G\alpha_{q}$) is indicated in red, identical flanking residues in blue, and flanking residues that result in notable charge differences are indicated in green. "*" denotes identical amino acid residues; ":" denotes highly conserved residues; "." denotes conserved residues.