Molecular Biology of *Pasteurella multocida* Toxin

Joachim H. C. Orth and Klaus Aktories

Abstract *Pasteurella multocida* toxin (PMT) is the causative agent of progressive atrophic rhinitis in swine. The 146 kDa single-chain toxin harbours discrete domains important for receptor binding, internalisation and biological activity. The molecular basis of the toxin's activity is the deamidation of a specific glutamine residue in the α-subunit of heterotrimeric G proteins. This results in an inhibition of the inherent GTPase activity leading to a constitutively active phenotype of the G protein. Due to the ability of the toxin to act on various families of heterotrimeric G proteins, a large subset of signal transduction pathways is stimulated.

Contents

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Pasteurella multocida is an opportunistic pathogenic bacterium living in the nasal pharyngeal space of animals. Infections of humans normally arise from scratches and bites by domesticated animals such as cats and dogs. Under special conditions infection of pigs with *P. multocida* leads to an atrophic rhinitis, which is characterised by the atrophy of nasal turbinate bones accompanied by a shortening and twisting of the snout. The causative agent of the atrophic rhinitis was found to be the bacterial protein toxin PMT. The toxin is produced by two (A and D) of five serogroups of *P. multocida* (Frandsen et al. 1991; Williamson 1994).

After entering the cell, the 146 kDa toxin activates various signal transduction pathways by stimulating heterotrimeric G proteins of the $G\alpha_{q/11}$, $G\alpha_{12/13}$ and $G\alpha_{i}$ family. After a discussion of the structure of PMT, the mechanisms by which PMT enters eukaryotic cells and produces biological effects will be described in detail.

1 Structure of PMT

Similar to many bacterial protein toxins, *Pasteurella multocida* toxin (PMT) is a multifunctional protein comprising different domains with specific functions. The toxin contains a receptor binding and translocation domain (B) and a biologically active (A) domain. Therefore, it can be described as a typical AB-type protein toxin (Fig. 1).

PMT consists of 1285 amino acid residues resulting in a mass of 146 kDa (Buys et al. 1990; Petersen 1990). The receptor binding domain is located in the N-terminal part of the protein, including the amino acid residues 1–580. Within this domain a putative translocation domain was found between residues 402–457. The N-terminus of PMT has homology ($\sim$20%) to another family of bacterial protein toxins named cytotoxic necrotizing factors found in uropathogenic *E. coli* (CNF1, CNF2, CNF3) and *Yersinia pseudotuberculosis* (CNFY) (Lemichez et al. 1997; Pullinger et al. 2001). CNFs are deamidases, which act on small GTPases like Rho, Rac or Cdc42 (Flatau et al. 1997; Schmidt et al. 1997). Consistent with this homology, the receptor binding domains of CNF and PMT are located in the N-terminal part and the active domains in the C-terminus (Busch et al. 2001; Pullinger et al. 2001).

The crystal structure of the C-terminal part of PMT revealed three domains designated C1, C2 and C3 (PDB ID 2EBF). The C3 domain is of major importance because it harbours the catalytic activity of the toxin to modify intracellular targets. The overall structure of the crystallised portion was described as Trojan horse-like shape with feet (C1), a body (C2) and a head (C3) (Kitadokoro et al. 2007; Miyazawa et al. 2006).

The feet C1-domain, encompassing amino acid residues 575–719, consists of seven helices. The tertiary structure of the first four helices shows similarity to the N-terminal portion of *Clostridium difficile* toxin B (25% identity) (Kamitani et al. 2010). In toxin B, these four helices serve as a plasma membrane targeting signal. An ectopically expressed GFP-fusion of a C1 fragment of PMT (amino acid

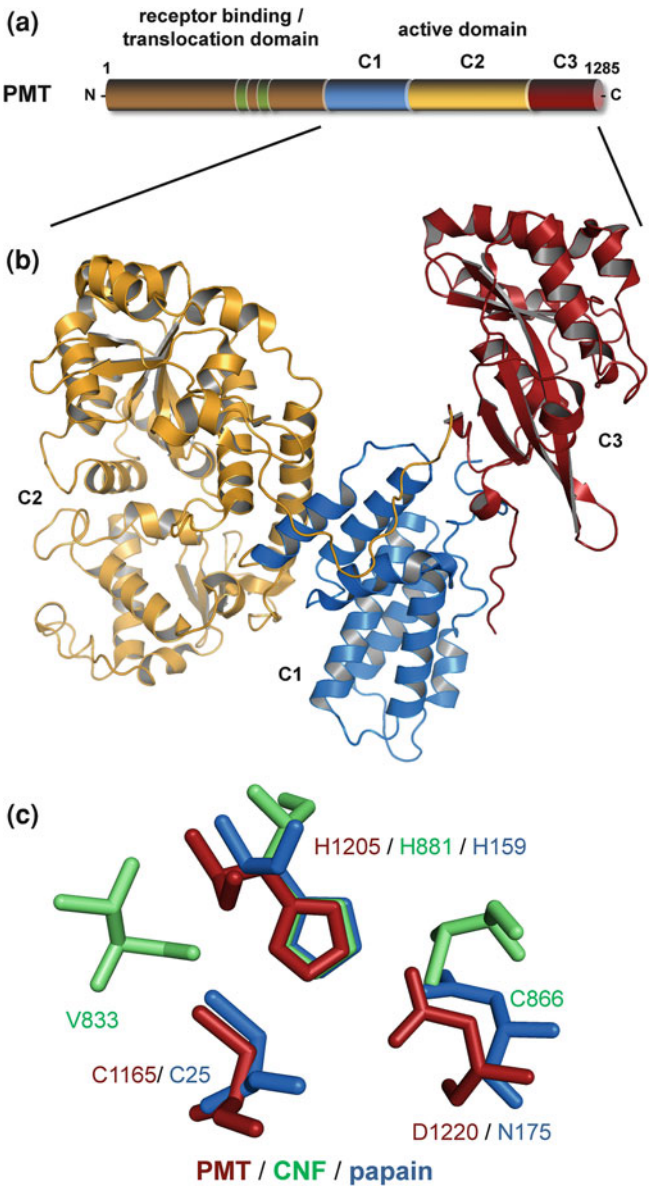

Fig. 1 Overall domain structure of PMT. **a** The toxin consists of an active domain in the C-terminus and a receptor binding and translocation domain in the N-terminus. **b** The crystal structure of a C-terminal fragment shows three distinct domains C1, C2 and C3. C1 is involved in plasma membrane binding. The function of C2 is still unknown and C3 is a deamidase to activate G protein α-subunits. **c** Comparison of the active sites of PMT, CNF1 and papain. Images were generated using PyMol and PDB data files 2EBF (C-PMT), 2EC5 (C-PMT^{C1159S}), 1HQO (CNF1) and 1POP (papain)

residues 569–671) shows strong localisation to the plasma membrane. Deletion of each of the four helices impaired localisation to the membrane. Additionally, a GFP-fusion of a C-terminal fragment of PMT (amino acid residues 569–1285) localised to the plasma membrane, whereas a deletion mutant of the first four helices of C1 (amino acid residues 671–1285) did not localise in the membrane. Because the primary target proteins of PMT are plasma membrane-bound heterotrimeric G proteins, impaired localisation to the substrate would diminish PMT toxicity. Congruently, expression of PMT fragments which are impaired in plasma membrane binding, showed no biological activity. Interestingly, an N-myristoylation peptide tag compensated for the deleted helices and restored PMT activity (Kamitani et al. 2010).

Moreover, this domain was identified as a conserved membrane localisation domain in clostridial glucosyltransferase toxins from *C. difficile*, *C. novyi*, *C. sordellii* or *C. perfringens* and the Multifunctional Autoprocessing RTX toxins (MARTX) from *Vibrio cholerae*, *V. vulnificus* or *V. anguillarum* (Geissler et al. 2009). GFP fusion proteins of these homologue domains are also membrane associated. Three amino acid residues were identified, which are 100% identical between membrane localisation domains. For PMT, these residues are Tyr-611, Ser-651 and Arg-653. Consequences of site-directed mutagenesis of these residues were only tested in MARTX-derived domains, but not in the correspondent PMT domain. The results showed that only the Ser and the Arg are essential for appropriate membrane targeting. Their function could be the maintenance of the overall structure of the four-helix bundle (Geissler et al. 2010).

The largest domain in the C-terminal part of PMT is the so-called body or C2 domain (amino acid residues 720–1,104). C2 consists of 18 helices and nine β-strands and can be divided into two subdomains. Both subdomains exhibit a structure typical of nucleotide-binding proteins. Folylpolyglutamate synthetase and cdc14bs show structural homology to the second subdomain. Because both enzymes interact with a phosphate group it was supposed that this could be a hint to the still unknown function of the C2 domain (Kitadokoro et al. 2007).

Intracellularly expressed PMT and toxin truncations confirmed that the biologically active domain, i.e. the G protein-activating domain is located within C3 (Aminova et al. 2008). This C3 domain (amino acid residues 1,105–1,285) is connected by a long loop (1,087–1,104) to the C2 domain. C3 is separated into two subdomains and provides the catalytic cleft for the enzymatic function of the toxin. Interestingly, a disulfide bond was found in C3 between Cys-1159 and Cys-1165. Mutational studies of these residues revealed that the toxic activity of PMT strictly depends on Cys-1165 but not Cys-1159 (Busch et al. 2001; Kitadokoro et al. 2007; Ward et al. 1998). In addition, His-1205, His-1223, Asp-1220 and Gln-1225 are essential for PMT activity (Kitadokoro et al. 2007; Orth et al. 2003; Pullinger and Lax 2007). Structural analysis of PMT mutants in which the disulfide bonds were ablated by replacing the respective Cys-1159 or Cys-1165 residues by Ser (PDB ID 2EC5/2EBH) displayed different structures compared to wt-PMT (PDB ID 2EBF). Most striking is a reorientation of Cys-1165 when Cys-1159 is replaced by Ser. Cys-1165 is displaced towards the catalytic cleft of the C3 domain and forms

a thiolate imidazolium ion pair with His-1205. The secondary structure of the reoriented C3 domain of $PMT^{Cys1195Ser}$ shows homology to the cysteine protease AvrPphB from *Pseudomonas syringae*, which in turn has structural similarity to the papain-like cysteine protease fold (Kitadokoro et al. 2007). The catalytic triad of PMT consists, therefore, of Cys-1165, His-1205 and Asp-1220 (Kitadokoro et al. 2007). The structure of the catalytic triad is very similar to the catalytic triads of transglutaminases like Factor XIII (PDB ID 1GGT) or fish derived transglutaminases (PDB ID 1GOD), as shown by superimposing the catalytic His residues. Additionally, PMT and papain exhibit similar topography of their catalytic triads (Fig. 1) (Orth et al. 2009). Despite the fact that PMT is a deamidase but not a transglutaminase nor thiol protease, it is not contradictory that the toxin shares the topography of the active sites of these enzymes. Transglutaminases replace the NH_2-group of the amide of glutamine by another amine group. Deamidases catalyse a similar type of chemical reaction, converting the amide into a carboxylate utilising water instead of an amine. Finally, transglutaminases perform the inverse reaction to thiol proteases. Therefore, the biological activity of PMT is supported by the crystal structure of the C3 domain.

The contribution of His-1223 and Gln-1225, which are also essential for toxin activity, is not completely understood to date. Both amino acid residues are in close proximity to the catalytic triad, and at least for Gln-1225, a direct participation in enzymatic activity was proposed (Kitadokoro et al. 2007).

2 Uptake into Eukaryotic Cells

Knowledge of the uptake of PMT into eukaryotic cells is limited. Three major steps are generally involved in the entry of bacterial protein toxins into their target cells. (i) Binding of a cell membrane receptor. (ii) Internalisation by endocytosis. (iii) Release of the biologically active component or the complete toxin into the cytosol (Sandvig and van Deurs 2005). For receptor binding and translocation, the N-terminal part of PMT is responsible (amino acids 1–580). Whereas the translocation domain is narrowed down to amino acid residues 402–457, the receptor binding region is not specified (Baldwin et al. 2004).

The PMT receptor is not yet characterised. Previous studies with colloidal, gold-labelled toxin observed binding to Vero cells. Incubation of mixed gangliosides with PMT inhibited toxin binding to Vero cells, indicating a competition between the added gangliosides and the PMT receptor. Therefore, it was suggested that PMT interacts with a ganglioside-type receptor (Pettit et al. 1993). More recently, these results were challenged by new findings. Using different approaches such as TLC-overlay, surface plasmon resonance analysis or toxin pull-down of liposomes, binding of PMT to membrane phospholipids like sphingomyelin and phosphatidylcholine was detected (Brothers et al. 2011). In contrast to the previous study, no binding of PMT to gangliosides like $GM_{1/2/3}$ was found. Interestingly, an interaction of a C-terminal fragment of PMT encompassing the crystallised

domains C1, C2 and C3 with GM_1 was discovered. The authors suggested that removal of the PMT N-terminus unmasks the membrane binding site of the C1 domain.

Surface plasmon resonance analysis leads to an at least bi-phasic binding of PMT to cells. After initial binding with low affinity to an abundant membrane component, a more specific binding to sphingomyelin could follow. The involvement of an additional proteinaceous receptor, which would induce endocytosis, was proposed (Brothers et al. 2011).

After binding to the receptor at the plasma membrane, the toxin enters the cell by endocytosis. Utilising various inhibitors of vesicle trafficking and GFP-fusions of PMT fragments, the intracellular pathway of endocytosed toxin was followed. GFP-fusions of the N-terminal portion of PMT harbouring the putative receptor binding and translocation domain were found to colocalize with transferrin receptor and at early time points with cholera toxin B subunit, indicating localisation in early endosomes (Repella et al. 2011). After passing early endosomes, transferrin receptor traffics to recycling endosomes and cholera toxin via the Golgi apparatus to the endoplasmic reticulum. Conversely, PMT is supposed to translocate to late endosomes where the translocation to the cytosol occurs (Repella et al. 2011). In line with this model, inhibitors of trafficking between Golgi apparatus and endoplasmic reticulum, like brefeldin A, do not inhibit the biological activity of PMT. However, cell entry depends on Arf6. The small GTPase Arf6 binds to endosomes and is important for the trafficking of recycling endosomes (Peters et al. 1995). Both a dominant-negative form and a constitutively cycling mutant of Arf6 inhibited PMT intoxication, implicating the involvement of this GTPase in toxin uptake (Repella et al. 2011).

It is thought that PMT translocates on the way from early to late endosomes to the cytosol. The acidification of the endosome plays a pivotal role for translocation (Baldwin et al. 2004; Rozengurt et al. 1990). Consequently, inhibition of endosome acidification by blockade of the vacuolar H^+ ATPases using bafilomycin A1 represses toxin activity (Baldwin et al. 2004). In addition, the direct transfer of plasma membrane bound toxin to the cytosol is inducible by mimicking the endocytic conditions, e.g. applying acidic medium to cells (Baldwin et al. 2004). The translocation depends on a putative translocation T-domain, consisting of two predicted hydrophobic helices (residues 402–423 and 437–457) linked by a peptide loop (residues 424–436). It is proposed that acidification induces a structural change in the toxin, which was previously observed utilising circular dichroism and measuring susceptibility to proteases (Smyth et al. 1995, 1999). This structural change exposes the T-domain, allowing it to insert into the vesicular membrane. Mutational studies of amino acid residues in the peptide loop between the hydrophobic helices suggest that acidic residues in this region are of major importance for membrane insertion (Baldwin et al. 2004).

So far, our knowledge of the membrane translocation process itself is scant. It is suggested that the toxin at least partly unfolds. Whether the toxin spontaneously refolds in the cytosol or whether chaperons support this process is not known.

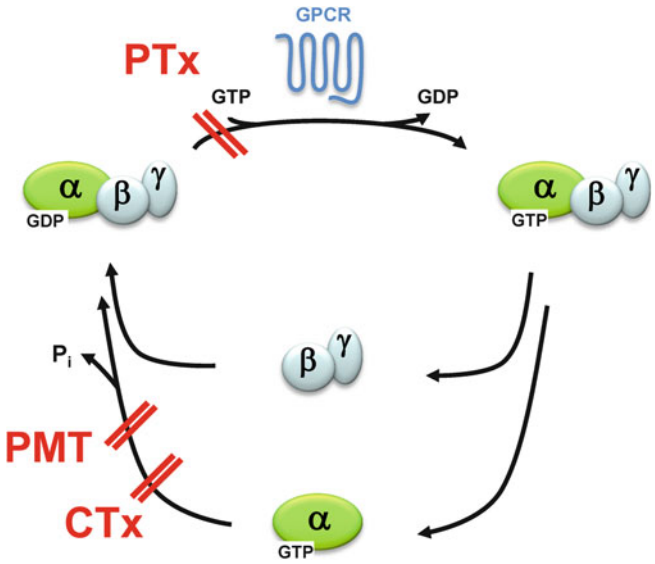

Fig.2 GTPase cycle of heterotrimeric G proteins. Inhibition of the GTPase cycle by PMT, cholera toxin (CTx) and pertussis toxin (PTx) at specific steps within the cycle is indicated

3 Activation of Heterotrimeric G Proteins

PMT activates signal transduction pathways, which are downstream of heterotrimeric G proteins. Heterotrimeric G proteins are molecular switches cycling between a GTP- and GDP-bound state (Bourne et al. 1990; Gilman 1987; Oldham and Hamm 2006, 2008; Sprang 1997). The inactive heterotrimeric complex of the α- and the non-dissociable βγ-subunit binds GDP. An agonist-activated heptahelical receptor, also known as G protein-coupled receptor (GPCR), functions as a guanine nucleotide exchange factor (GEF). The activated GPCR induces conformational changes in the α-subunit to release the GDP and to rebind GTP (Gilman 1987; Rasmussen et al. 2011; Westfield et al. 2011). After binding GTP, the heterotrimeric complex dissociates into the GTP-bound α-subunit and the βγ-subunit. Both can interact and stimulate their own effectors. The inherent GTPase activity of the α-subunit, which can be stimulated by regulators of G protein signalling (RGS) (Hollinger and Hepler 2002; Ross and Wilkie 2000), hydrolyses the γ-phosphate of GTP. The GDP-bound α-subunit reassociates with Gβγ and signal transduction is terminated (Fig. 2). The heterotrimeric G proteins are grouped with respect to the effector interactions of the α-subunits: The G_s family activates the adenylyl cyclase (Northup et al. 1980), $G_{i/o}$ proteins inhibit the adenylyl cyclase (Bokoch et al. 1983), $G_{q/11}$ stimulates the phospholipase C (PLC) β1 (Taylor et al. 1990) and $G_{12/13}$ activates the small GTPase Rho via RhoGEF proteins (Birnbaumer 2007a, b; Hepler and Gilman 1992; Riobo and Manning 2005).

PMT activates diverse family members of heterotrimeric G proteins. Via $G\alpha_q$ the toxin leads to activation of PLCβ1, resulting in increased levels of diacylglycerol, inositoltrisphosphate and Ca^{2+} (Rozengurt et al. 1990; Staddon et al. 1991; Wilson et al. 1997; Zywietz et al. 2001). PMT-activated $G\alpha_{13}$ leads to stimulation of the small GTPase RhoA (Orth et al. 2005; Zywietz et al. 2001) and via $G\alpha_i$ the adenylyl cyclase is inhibited (Orth et al. 2008). A comprehensive overview of PMT-induced signalling is lined out in Cellular effects of Pasteurella multocida toxin by Wilson.

The molecular mechanism by which PMT activates signalling via heterotrimeric G proteins was elucidated on the basis of $G\alpha_i$. One advantage of $G\alpha_i$ is the possibility to easily determine the GTPase activity, which is the key in terminating G protein signalling. A so-called multiple cycle GTPase assay (Aktories and Jakobs 1981) in membrane preparations was utilised to measure the effect of PMT on heterotrimeric G proteins (Orth et al. 2008). An agonist of the G_i-coupling EDG-receptor was used to induce G protein cycling and the released γ-phosphate was measured. The receptor agonist LPA exhibited a strong induction of GTPase activity of $G\alpha_i$. Interestingly, in membranes of PMT-intoxicated cells the basal GTPase activity was diminished and no increase of GTPase activity was induced by receptor agonists (Orth et al. 2008). The inhibition of GTPase activity induced by PMT suggested that the toxin activated G protein signalling by blocking the terminating GTP hydrolysis. However, the multiple cycle GTPase assay was not appropriate to determine unequivocally the step of GTP hydrolysis. Besides inhibition of the GTPase activity, the toxin caused uncoupling of $G\alpha_i$ from its receptor. This was measured by receptor-induced GTPγS-binding, which was blocked by PMT treatment (Orth et al. 2007).

The effect of PMT was compared to that of pertussis toxin (PTx). PTx ADP-ribosylates $G\alpha_i$ proteins and inhibits the interaction of the heterotrimeric G protein with the GPCR (Gierschik 1992; Katada and Ui 1982; Murayama and Ui 1983; Nürnberg 1997; Ui 1984). Thereby the activation and the cycling of the G protein are blocked. The observed outcome is the inhibition of the GTPase activity and receptor-induced GTPγS-binding. In respect to the effects measured (e.g., inhibition of steady-state GTP hydrolysis and blockade of agonist-induced GTPγS-binding), PMT and PTx caused similar results, however, PTx blocked $G\alpha_i$ signalling and PMT stimulated $G\alpha_i$ signalling as revealed by inhibition of the adenylyl cyclase. This indicated that PMT disrupts GTPase cycling at a different step as PTx.

A major experimental advantage of using $G\alpha_i$ compared to other G protein α-subunits is the possibility of expressing the functional recombinant protein in high amounts. Therefore, $G\alpha_{i2}$ was coexpressed with the toxin in *E. coli*. Subsequently, the purified G protein was utilised for a single turnover GTPase assay. This kind of assay enables the determination of the GTPase reaction itself and not only the complete cycle (e.g., nucleotide-binding, hydrolysis and release). The intrinsic GTPase activity of $G\alpha_{i2}$, which was coexpressed with the inactive PMT mutant Cys-1165-Ser, was stimulated by addition of regulator of G protein signalling (RGS)3s or RGS16. Both RGS proteins function as GTPase activating proteins

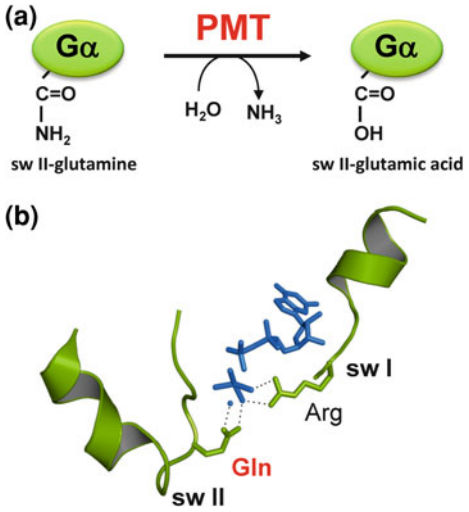

Fig. 3 Molecular mechanism of PMT to activate heterotrimeric G proteins. The toxin deamidates a specific glutamine residue in the α-subunit of heterotrimeric G proteins leading to a glutamic acid residue in this position. **a** Structural overview of the switch (sw) I and II region in $G\alpha_{i1}$ binding to GDP-AlF_4^-, mimicking the transition state complex of the hydrolysis of the γ-phosphate. The conserved Gln and Arg residues participate in the coordination of the γ-phosphate and the attacking water nucleophile (*blue spot*). Image was generated using PyMol and PDB data file 1GFI

(GAP) to facilitate the intrinsic GTPase reaction of the α-subunit. $G\alpha_{i2}$ coexpressed with active PMT showed decreased basal GTP-hydrolysing activity and RGS proteins did not increase this activity over the basal level (Orth et al. 2009). These results defined the hydrolysis of GTP as the critical step inhibited by the toxin's action.

Tandem mass spectrometric analysis of recombinant $G\alpha_{i2}$, which was coexpressed with PMT, revealed a 1 Da change in the switch II region of the α-subunit. A peptide of the switch II region, encompassing amino acid residues 199-MFDVGG**Q**R-206, was recovered after tryptic digestion and MS analysis of $G\alpha_{i2}$ coexpressed with inactive toxin. The corresponding peptide of $G\alpha_{i2}$ coexpressed with active toxin was also found. Additionally, a second peptide with a 1 Da higher mass appeared. Tandem MS analysis revealed the following peptide: 199-MFDVGG**E**R-206. Hence, the toxin deamidates Gln-205 of $G\alpha_{i2}$ resulting in glutamic acid in this position (Fig. 3).

The glutamine residue in the switch II region is conserved throughout all α-subunits of heterotrimeric G proteins. This Gln stabilises the pentavalent transition state of the GTP hydrolysis and coordinates the water nucleophile attacking the γ-phosphate of the nucleotide (Coleman et al. 1994; Sprang 1997; Tesmer et al. 1997). Mutations of this site are commonly known to inhibit the GTPase activity consequently leading to a constitutive activation of G protein signalling

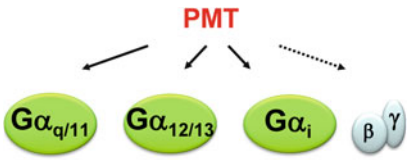

Fig. 4 Heterotrimeric G proteins activated by PMT. The toxin acts on the $G_{q/11}$-, $G_{12/13}$- and G_i-family to induce their signal transduction via their appropriate effectors. By activating the α-subunit the corresponding βγ-subunit is released and stimulates its own signalling cascade

(De Vivo et al. 1992). This is true not only for the α-subunits of heterotrimeric G proteins, but also for small GTP-binding proteins of the Ras superfamily (Bourne et al. 1989). Another important aspect of this type of mutation of G proteins is their frequent finding in mammalian tumours. As known for the transforming Ras mutation at position Gln-61, mutations of the α-subunit of heterotrimeric G proteins, causing inhibition of the GTPase activity, were described as transforming oncogenes and were found in diverse types of tumours (Kalinec et al. 1992; Radhika and Dhanasekaran 2001). For example, mutations of $G\alpha_{i2}$ at position Gln-205 have been observed in pituitary adenomas and mutations of Gln-209 of $G\alpha_q$ were found in melanoma of the uvea and blue naevi (Van Raamsdonk et al. 2008; Williamson et al. 1995).

To further verify that an exchange of the conserved Gln to Glu, as catalysed by PMT, blocks GTP hydrolysis, the mutation was introduced in $G\alpha_{i2}$ (Gln-205) and $G\alpha_q$ (Gln-209). Both mutations constitutively activated the G proteins leading to an inhibition of adenylyl cyclase or stimulation of PLCβ1, respectively (Orth et al. 2009). All these data show that PMT deamidates an essential Gln residue in the switch II region of the α-subunit of heterotrimeric G proteins. The resulting Glu residue is not capable of hydrolysing the bound nucleotide, leading to a constitutive active phenotype of the G protein.

Another amino acid residue contributing to the γ-phosphate coordination of GTP is the Arg in the switch I region. Interestingly, this Arg in the switch I region of $G\alpha_s$ is the target site for ADP-ribosylation by cholera toxin (Dop Van et al. 1984; Freissmuth and Gilman 1989), a key feature of cholera pathogenesis. Accordingly, the GTPase activity of CTx-modified $G\alpha_s$ is blocked and the adenylyl cyclase is stimulated by constitutively active $G\alpha_s$ (Cassel and Selinger 1977).

4 Substrate Specificity

As mentioned above, the PMT-targeted Gln in the switch II region of heterotrimeric G protein α-subunits is conserved throughout all members of G proteins. However, the toxin-induced activation has been verified for only a subset of heterotrimeric G proteins. Initially, the activation of heterotrimeric G proteins by PMT was studied indirectly by measurement of the specific downstream signalling

of the respective G proteins (Higgins et al. 1992; Rozengurt et al. 1990; Staddon et al. 1990, 1991). By different methods, members of the $G\alpha_q$-, $G\alpha_{13}$- and $G\alpha_i$-family have been identified as substrates of the toxin. Identification of the primary molecular mode of action of PMT as a deamidation reaction allows the direct study of the covalent modification of toxin-targeted G proteins (Fig. 4).

Most importantly, the deamidation can be verified by mass spectrometric analysis of recombinant protein for $G\alpha_{i2}$. Moreover, the shift of the isoelectric point (pI), resulting from the deamidation of the Gln to the more acidic Glu, is detectable by 2D-gel electrophoresis. For example, a change of 0.07 units was calculated and verified for $G\alpha_{i2}$ and $G\alpha_{i1}$, which were recovered from PMT-treated cells (Orth et al. 2009).

$G\alpha_q$ is a well-known target of PMT (Wilson et al. 1997). Several biochemical data support this view. First, as mentioned above, native gel electrophoresis demonstrates a shift of $G\alpha_q$ after PMT intoxication, indicating a change of the pI induced by deamidation (Orth et al. 2009). More directly, deamidation of $G\alpha_q$ was verified by a monoclonal antibody, which specifically detects the switch II region of $G\alpha_q$ covering the deamidated Gln-209 (i.e. Glu-209) (Kamitani et al. 2011). Because the monoclonal antibody discriminates perfectly between unaffected G proteins and PMT-deamidated G proteins, it is a useful tool to verify the toxin's action at the G protein level. This antibody was employed to answer the puzzle of PMT substrate specificity, which mystified studies with the toxin for years. Thus, several previous studies using mouse embryonic fibroblasts deficient for $G\alpha_q$, $G\alpha_{11}$ or both, showed that PMT-induced $G\alpha_{q/11}$-dependent signalling to the PLCβ1 exclusively via $G\alpha_q$ but not via $G\alpha_{11}$ (Orth et al. 2004; Zywietz et al. 2001). These findings suggested that $G\alpha_{11}$ was not modified by PMT, although the switch II region shares high homology between all heterotrimeric G proteins and is even identical between $G\alpha_q$ and $G\alpha_{11}$ (Orth et al. 2004; Zywietz et al. 2001). However, the new findings obtained by deamidation-specific antibody and now confirmed by MS analysis in our laboratory (J.H.C. Orth and K. Aktories, unpublished data) indicate that both $G\alpha_q$ and $G\alpha_{11}$ are deamidated by PMT. These results suggest that differences in toxin-activated $G\alpha_{q/11}$-signalling are not based on the toxin-substrate interaction per se, but possibly on the interaction of the deamidated G proteins ($G\alpha_q$ and $G\alpha_{11}$) with their effectors.

As for $G\alpha_{q/11}$, cellular effects or signal transduction events were utilised to demonstrate PMT-induced activation of the $G\alpha_{12/13}$ family. A common effector of $G\alpha_{12/13}$ and $G\alpha_{q/11}$ is the small GTPase RhoA (Vogt et al. 2003). RhoGEF proteins, which can be exclusively activated by $G\alpha_{12/13}$ (p115RhoGEF), $G\alpha_{q/11}$ (p63RhoGEF) or by both (LARG) connect the heterotrimeric G proteins with the small G protein RhoA (Booden et al. 2002; Hart et al. 1998; Kozasa et al. 1998; Lutz et al. 2005). Consequently, PMT treatment of cells leads to RhoA-dependent reorganisation of the actin cytoskeleton, stress fibre formation and downstream, to an increase in endothelial permeability (Dudet et al. 1996; Essler et al. 1998; Lacerda et al. 1996; Zywietz et al. 2001). Utilising mouse embryonic fibroblasts deficient for $G\alpha_{q/11}$ or $G\alpha_{12/13}$, the involvement of $G\alpha_{12/13}$ besides $G\alpha_{q/11}$ in PMT-induced RhoA activation was demonstrated (Zywietz et al. 2001). Another method

of differentiating between $G\alpha_{q/11}$- and $G\alpha_{12/13}$-mediated signalling is the cyclic peptide YM-254890, which is a specific inhibitor of $G\alpha_{q/11}$ signalling (Takasaki et al. 2004). Using both approaches (e.g., genetic knock-out and YM-254890), the dissection of PMT-induced G protein signalling is possible. Most convincingly, the rescue of $G\alpha_{13}$ in $G\alpha_{12/13}$-deficient cells treated with the $G\alpha_{q/11}$ inhibitor reconstitutes toxin-induced RhoA activation (Orth et al. 2005).

Taken together, the activation of heterotrimeric G proteins by PMT is demonstrated for $G\alpha_i$, $G\alpha_q$ and $G\alpha_{13}$ by measuring G protein signalling. Studies on the G protein itself show deamidation of $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_q$ by a deamidation-triggered change of the pI or $G\alpha_q$ and $G\alpha_{11}$ by recognition by a specific antibody. The most stringent evidence for PMT-induced activation is MS analysis. Unfortunately, up to now this has been described only for $G\alpha_{i2}$.

5 G Protein Prerequisites for PMT Action

5.1 Role of Gβγ

Heterotrimeric G proteins consist of the nucleotide-binding α-subunit and the non-dissociable βγ-subunit (Gilman 1987; Oldham et al. 2006; Sprang 1997). After GPCR-induced activation and subsequent dissociation of Gα from Gβγ, both parts induce downstream signalling. Therefore, various questions arise concerning PMT action and Gβγ: (i) Does PMT induce dissociation of the heterotrimeric complex? (ii) Is Gβγ signalling stimulated by PMT? (iii) Does the cycling between heterotrimeric complex and the dissociated form influence the activation by PMT?

Classical studies have shown that the heterotrimeric complex and not the free α-subunit of $G\alpha_i\beta\gamma$ is the preferred substrate of PTx, allowing the determination of the formation of the heterotrimeric complex by PTx-catalysed ADP-ribosylation (Katada et al. 1986). Treatment of mammalian cells with PMT as well as coexpression of $G\alpha_{i2}$ with PMT leads to an inhibition of PTx-induced ADP-ribosylation of $G\alpha_i$. Additionally, the binding of Gβγ to $G\alpha_{i2}$, coexpressed with active toxin, is reduced to the same extent as $G\alpha_{i2}$ is deamidated by PMT ($\sim$50%) (Orth et al. 2008, 2009). Therefore, a dissociation of the heterotrimeric complex by PMT is likely.

PMT-induced activation of Gβγ signalling is most convincingly demonstrated by determination of the activity of PI3Kγ (Preuss et al. 2009). PI3Kγ is an established effector of βγ-subunits leading to PIP_3 formation (Maier et al. 1999; Stephens et al. 1997). Therefore, PMT induces the translocation of a GFP sensor protein (GFP-$Grp1_{PH}$), which interacts with PIP_3, to the plasma membrane (Preuss et al. 2009). Moreover, using scavengers of Gβγ, like GRK2-CT (G-protein-coupled-receptor kinase 2 C-terminus) (Wu et al. 1998), the direct involvement of Gβγ in PMT-induced PI3Kγ activation can be demonstrated.

Sequestration of βγ-subunits not only blocks Gβγ downstream signalling, but also activation of Gα-dependent signalling by PMT. Overexpression of GRK2-CT or phosducin, both proteins that sequester Gβγ (Hawes et al. 1994; Wu et al. 1998), strongly reduces PMT activation of Gα-dependent signalling (Preuss et al. 2009). Moreover, two mutants of $G\alpha_q$ (I25A/E26A and G208A) were utilised to study the role of Gβγ in more detail. The mutants manipulate the interaction of Gα with Gβγ. The double mutant of $G\alpha_q$ (I25A/E26A) does not bind Gβγ, while $G\alpha_q^{G208A}$ binds with increased affinity to Gβγ (Jetzt et al. 2003; Lee et al. 1992). Because both mutants inhibit PMT-induced $G\alpha_q$ activation, it is likely that the binding capability of Gα to Gβγ itself is not important, but rather the cycling of the heterotrimeric complex, e.g. the association and dissociation of Gβγ. Therefore, it has been suggested that during cycling of the G protein a favourable structure serves as target for the toxin (Preuss et al. 2009). Furthermore, Kamitani et al. tested in an in vitro system whether PMT prefers the monomeric Gα or the heterotrimeric complex Gαβγ for deamidation (Kamitani et al. 2011). These studies showed that both states are recognised by the toxin, but the monomeric α-subunit is two orders of magnitude less sensitive than the heterotrimeric complex. Thus, the presence of Gβγ at least enhances PMT action. On the other hand, toxin-induced deamidation during coexpression in *E. coli* occurs without Gβγ (Orth et al. 2009), indicating that Gβγ is not absolutely required for modification of the α-subunit by PMT.

5.2 PMT-Induced Activation of G Proteins is Independent of GPCR Interaction

The interaction, i.e. activation of heterotrimeric G proteins by GPCR, depends mainly on the C-terminal amino acid residues of the Gα protein. The last five amino acids are essential for interaction and determine the specificity of G protein-GPCR coupling (Conklin et al. 1993; Hamm 1998; Parekh 2006). On the basis of $G\alpha_q$ it was studied whether PMT-induced activation depends on any receptor interaction (Orth et al. 2007). Therefore, G protein constructs, which cannot couple to GPCRs or G protein-receptor fusion proteins, were tested for PMT-induced activation. As expected, the C-terminal deletion mutant of $G\alpha_q$ was not stimulated by a Gq-coupling receptor. In contrast, the toxin was still able to stimulate this deletion mutant, indicating that the function of PMT is independent of the coupling of the G protein with GPCRs. Vice versa, it was tested whether the toxin also activates an α_{1b}-adrenoceptor-$G\alpha_q$ chimera. This chimera was also stimulated by the toxin, supporting the view that PMT acts completely independently of any receptor interaction (Orth et al. 2007).

In agreement with these results, it was shown that PTx does not block PMT-induced $G\alpha_i$ activation (Orth et al. 2008). PTx ADP-ribosylates a Cys residue in the C-terminus of $G\alpha_{i/o}$ proteins and inhibits interaction of the G protein with the

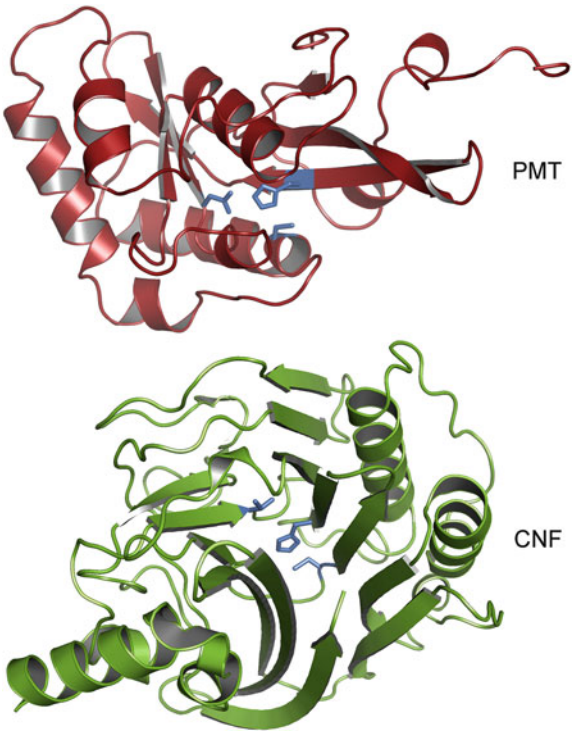

Fig. 5 Comparison of the catalytic domains of PMT and CNF1. Crystal structures of the catalytic active domains of PMT and CNF1. Catalytic triads (*blue sticks*) of PMT and CNF1 are indicated in the folds. Images were generated using PyMol and PDB data files 2EC5 (C-PMT^{C1159S}) and 1HQO (CNF1)

receptor (Katada et al. 1982; Murayama et al. 1983). Even after PTx intoxication, PMT is able to stimulate Gα_i leading to an inhibition of the adenylyl cyclase.

6 PMT and Cytotoxic Necrotizing Factors

PMT belongs to a large group of deamidating toxins and/effectors, which cause major pathophysiological alterations of target cells by removing the amide functional group from a specific glutamine residue of the targeted protein substrate. While the targets of PMT are the α-subunits of heterotrimeric G proteins, the cytotoxic necrotizing factors (CNF) 1–3 from *E. coli* and CNFY from *Y. pseudotuberculosis* deamidate small GTPases of the Rho family (Flatau et al. 1997; Hoffmann et al. 2004; Hoffmann and Schmidt 2004; Schmidt et al. 1997; Stoll et al. 2009). It is fascinating that CNFs target the functionally equivalent glutamine residue (e.g., Gln-61 of Rac and Cdc42 or Gln-63 of Rho) of GTPases, resulting in inhibition GTP hydrolysis and constitutive activation of the small G proteins as known for PMT and heterotrimeric G proteins (Aktories 2011).

PMT and CNFs share significant amino acid sequence homology in the N-terminal receptor binding and translocation domain (Lemichez et al. 1997;

Pullinger et al. 2001). However, in contrast to the functionally related eukaryotic targets and type of reaction, no homology is observed in the primary sequence or the tertiary structure of the catalytic active deamidase domain. Moreover, the topography of the catalytic centres of PMT and CNF1 are different (Fig. 5). The catalytic triads of PMT and CNF share the catalytic active Cys and His residues. In the case of PMT, the third catalytic active residue is an Asp and for CNF it is a Val. Superimposing the active His residues revealed that the catalytic triads of PMT and CNF do not match (Fig. 1c). All structural data together indicate that the molecular mechanism of PMT and CNFs to activate G proteins was independently developed during evolution.

7 Outlook

For more than 20 years after the discovery of PMT, its molecular mode of action remained enigmatic. With the elucidation of the toxin as a deamidase, a new era of PMT research has developed. There are still important questions to answer. The precise structural determinants of substrate (G protein) recognition by the toxin are not known. The uptake of the toxin is still not well understood. Are domains 1 and/or domain 2 of the Trojan horse of the C-terminal part of PMT involved in uptake or processing? The answers to these important questions will help to further understand the molecular biology of the toxin. Another important area which has still to be studied is the functional consequence of the actions of the toxin, especially with respect to its pathophysiological role and mechanism in disease. What are the precise pathophysiological steps finally leading to the sequelae of bone destruction of the nose? Thus, we have much to learn about PMT.

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