Pasteurella multocida toxin activation of heterotrimeric G proteins by deamidation

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***Pasteurella multocida* toxin is a major virulence factor of *Pasteurella multocida*, which causes pasteurellosis in men and animals and atrophic rhinitis in rabbits and pigs. The ≈145 kDa protein toxin stimulates various signal transduction pathways by activating heterotrimeric G proteins of the $G\alpha_q$, $G\alpha_i$, and $G\alpha_{12/13}$ families by using an as yet unknown mechanism. Here, we show that *Pasteurella multocida* toxin deamidates glutamine-205 of $G\alpha_{i2}$ to glutamic acid. Therefore, the toxin inhibits the intrinsic GTPase activity of $G\alpha_i$ and causes persistent activation of the G protein. A similar modification is also evident for $G\alpha_q$, but not for the closely related $G\alpha_{11}$, which is not a substrate of *Pasteurella multocida* toxin. Our data identify the α-subunits of heterotrimeric G proteins as the direct molecular target of *Pasteurella multocida* toxin and indicate that the toxin does not act like a protease, which was suggested from its thiol protease-like catalytic triad, but instead causes constitutive activation of G proteins by deamidase activity.**

bacterial protein toxin | Gi protein | posttranslational modification | transglutaminase

Heterotrimeric G proteins play key roles in signal transduction via heptahelical membrane receptors also known as G protein-coupled receptors (GPCRs). These heterotrimeric G proteins are also targets of bacterial protein toxins. Cholera toxin acts on G_s and locks $G\alpha_s$ in an active state by ADP-ribosylation of Arg-201, resulting in inhibition of the intrinsic GTPase activity (1). Pertussis toxin (PTx) ADP-ribosylates the C-terminal cysteine of $G\alpha_{i/o}$ (2, 3), thereby fixing the G protein in an inactive heterotrimeric $G\alpha\beta\gamma$ state, which is uncoupled from GPCR activation. It is generally accepted that ADP-ribosylation of G protein α-subunits by cholera toxin and pertussis toxin contribute to the pathophysiological mechanism of cholera and whooping cough, respectively.

Pasteurella multocida toxin (PMT) is another toxin that affects signaling via heterotrimeric G proteins. However, until now its molecular mode of action was not known. The protein toxin is a major virulence factor of *Pasteurella multocida*, which causes pasteurellosis in men and animals and progressive atrophic rhinitis of pigs and rabbits (4, 5). The latter disease is characterized by osteoclastic bone resorption of nasal turbinates, an effect that is uniquely caused by PMT. PMT activates a number of mitogenic signaling pathways, including MAP kinases (e.g., ERK) and JAK/STAT pathways (6, 7) and is one of the most potent known mitogens of fibroblasts (8, 9). Therefore, a role of PMT in tumor development and cancer has been proposed (10).

Intoxication of mammalian cells by PMT increases total inositol phosphate levels and mobilizes calcium signaling because of activation of phospholipase Cβ (PLCβ) (supporting information (SI) Fig. S1) (11, 12). Stimulation of PLCβ is caused by PMT-induced activation of $G\alpha_q$ (12). Studies using $G\alpha_{q/11}$-deficient mouse embryonic fibroblasts (MEFs) indicate that $G\alpha_q$, but not the closely related $G\alpha_{11}$, is activated by PMT (13, 14). In addition to $G\alpha_q$, PMT activates $G\alpha_{13}$ of the $G_{12/13}$ family, resulting in formation of stress fibers and activation of the small GTPase RhoA (15). More recently, we observed that PMT is also a potent activator of $G\alpha_i$, converting the G protein into a PTx-insensitive state (16). The latter finding is of special importance, because G_i proteins are readily accessible for analyses and recombinant expression.

PMT is a typical AB-toxin with a receptor binding and translocation domain at the N terminus and a biological active part at the C terminus (17, 18). A C-terminal fragment of PMT, which consists of 3 domains, covering amino acids 569 to 1285, has been crystallized (19). The structure revealed that the C-terminal C3 domain (residues 1105–1285), which defines the minimal intracellular activity domain responsible for activation of calcium and mitogenic signaling (20), resembles that of a papain-like protease. The domain harbors a catalytic triad characteristic of thiol proteases, consisting of the essential amino acids cysteine-1165 (17, 18), histidine-1205 (21) and aspartic acid-1220. However, no proteolytic activity by PMT has thus far been demonstrated.

Results

Effects of Coexpression of Recombinant $G\alpha_{i2}$ with PMT in *Escherichia coli* on ADP-Ribosylation by PTx and Interaction with $G\beta\gamma$. Because PMT did not exhibit any effect on its G protein targets in vitro, we decided to coexpress $G\alpha_{i2}$ and the biologically active C-terminal fragment of PMT (residues 581-1285; PMT-C^{wt}) in *E. coli* (Fig. S2). To monitor any action of PMT on $G\alpha_{i2}$ we studied ADP-ribosylation of G_i by PTx, because it was shown that treatment of intact cells with PMT leads to inhibition of PTx-induced ADP-ribosylation of G_i (16). When recombinant $G\alpha_{i2}$, which was coexpressed with the active PMT fragment, was isolated and incubated with PTx in the presence of radiolabeled NAD and $G\beta\gamma$, little or no ADP-ribosylation was detected (Fig. 1*A*). In contrast, $G\alpha_{i2}$ coexpressed with an inactive PMT mutant (PMT-C^{C1165S}) (17) was ADP-ribosylated by PTx (Fig. 1*A*), excluding unspecific effects of coexpression. Therefore, we concluded that PMT had acted on $G\alpha_i$ under the conditions of recombinant coexpression in *E. coli*.

The heterotrimeric $G\alpha_i\beta\gamma$ complex, and not the monomeric $G\alpha_i$, is the preferred substrate of PTx (22). To test whether the formation of $G\alpha_{i2}\beta\gamma$ is affected by coexpression of PMT and $G\alpha_{i2}$, the binding of $G\beta\gamma$ to recombinant $G\alpha_{i2}$ was assessed in a pull-down assay. To this end, $G\beta\gamma$ was transcribed and translated in vitro in the presence of [^{35}S]methionine. The resulting ^{35}S-labeled $G\beta\gamma$ was pulled down with GST-tagged $G\alpha_{i2}$, which was

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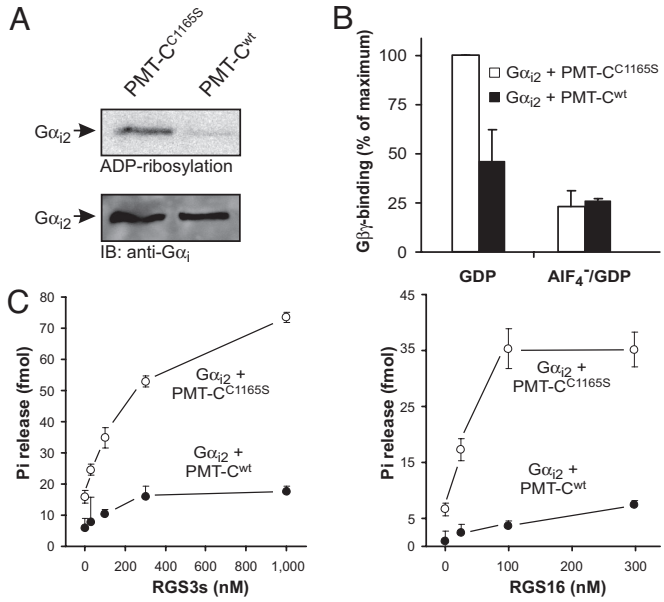

Fig. 1. Coexpression of $G\alpha_{i2}$ with PMT affects $G\beta\gamma$ binding and GTPase activity of the $G\alpha_{i2}$ protein. (*A*) $G\alpha_{i2}$ was coexpressed with inactive PMT fragment (PMT-C^{C1165S}) or active PMT fragment (PMT-C^{wt}). PTx-induced ADP-ribosylation of the resulting $G\alpha_{i2}$ was performed after addition of recombinant $G\beta_1\gamma_2$. (*Upper*) A representative autoradiogram of [^{32}P]-ADP-ribosylation and a representative immunoblot by using anti-$G\alpha_{i1-3}$ serum are shown. (*B*) The binding of $G\beta_1\gamma_2$ was measured by autoradiography of in vitro translated, [^{35}S]methionine-labeled $G\beta_1\gamma_2$ subunits pulled down with $G\alpha_{i2}$, coexpressed with inactive PMT-C^{C1165S} (white bars) or active PMT-C^{wt} (black bars) in the presence of GDP or GDP plus AlF_4^-. Shown is the relative binding of 3 independent experiments. (*C*) Single cycle GTPase activity of $G\alpha_{i2}$ coexpressed with inactive PMT-C^{C1165S} (○) or active PMT-C^{wt} (●). $G\alpha_{i2}$ was incubated with the indicated concentrations of RGS3s (*Left*) or RGS16 (*Right*), and single cycle GTPase activity was measured. Shown are representative results (mean ± SE, $n = 3$) from at least 3 independent experiments.

purified from coexpression with either active or inactive PMT-C. $G\alpha_{i2}$ obtained from coexpression with active PMT-C^{wt} exhibited diminished $G\beta\gamma$-binding in the presence of GDP compared with $G\alpha_{i2}$ obtained from coexpression with inactive PMT-C^{C1165S} (Fig. 1*B*). As controls, we studied $G\beta\gamma$-binding in the presence of AlF_4^-/GDP, which is known to reduce the affinity of $G\beta\gamma$ subunits to the $G\alpha$ subunit (23). This suggested that PMT-C modifies $G\alpha_{i2}$ so as to inhibit $G\alpha_{i2}$ interaction with $G\beta\gamma$ and thereby preventing ADP-ribosylation by PTx.

Effects of PMT on GTPase Activity of the G_i Protein. Next we studied the effects of PMT on the intrinsic GTPase activity of $G\alpha_{i2}$. The GTPase activity of $G\alpha_{i2}$ was stimulated in a single turnover assay by regulators of G protein signaling, RGS3s or RGS16 (Fig. 1*C*), both of which stimulate GTP hydrolysis by $G\alpha_{i/o}$ proteins (24). Whereas coexpression of the inactive PMT-C^{C1165S} with $G\alpha_{i2}$ had no effect on basal or RGS-stimulated GTPase activity, coexpression of $G\alpha_{i2}$ with the active PMT-C^{wt} fragment inhibited the RGS-stimulated GTPase activity. These data were in line with the view that a covalent modification of $G\alpha_{i2}$, which had occurred during coexpression of the active fragment of PMT with the $G\alpha_{i2}$ protein, resulted in inhibition of basal and RGS-stimulated GTP hydrolysis.

Deamidation of Recombinant $G\alpha_{i2}$ After Coexpression with PMT in *Escherichia coli*. To identify the PMT-induced structural changes to $G\alpha_{i2}$, we performed mass spectrometric (MS) analysis. In *E. coli*, $G\alpha_{i2}$ protein was coexpressed with active PMT-C^{wt} or inactive PMT-C^{C1165S}. Then the G protein was separated by SDS/PAGE, excised from the gel, and digested with trypsin. The tryptic peptides were analyzed by HPLC and tandem MS for peptide sequencing. A peptide, encompassing residues 199-MFDVGG**E**R-206, was detected from the $G\alpha_{i2}$ preparation obtained after coexpression with active PMT-C^{wt} that differed from the corresponding peptide (199-MFDVGG**Q**R-206) from $G\alpha_{i2}$ coexpressed with inactive PMT-C^{C1165S} at the glutamic acid-205 position. Thus, MS analysis indicated that after coexpression of $G\alpha_{i2}$ with PMT-C^{wt} glutamine-205 of $G\alpha_{i2}$ was specifically deamidated, resulting in a glutamic acid at this position (Fig. 2).

Because deamidation of glutamine alters the isoelectric point (pI) of the protein, we studied whether coexpression of $G\alpha_i$ with PMT in *E. coli* resulted in changes in the migration behavior of the $G\alpha_i$ protein on 2-D gels. In accord with the expected decrease in pI of 0.07, we observed a shift of $G\alpha_{i2}$ after

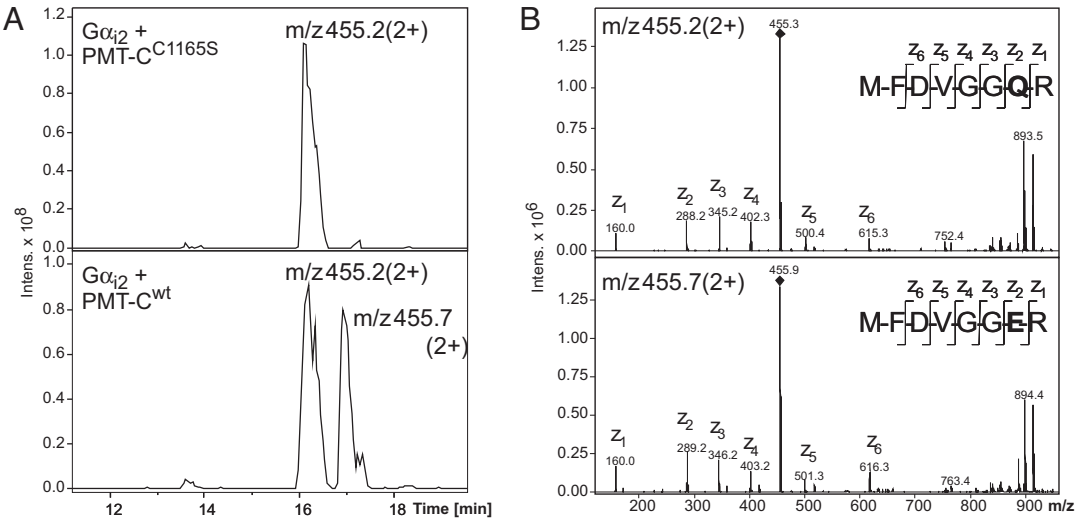

Fig. 2. Deamidation of $G\alpha_{i2}$ glutamine-205 by PMT. (*A*) Combined extracted ion chromatograms for *m/z* 455.2 and 455.7, corresponding to the tryptic peptides MFDVGGQR and MFDVGGER (amino acids 199–206) of $G\alpha_{i2}$, are shown. (*Upper*) $G\alpha_{i2}$ coexpressed with inactive PMT-C^{C1165S}. (*Lower*) $G\alpha_{i2}$ coexpressed with active PMT-C^{wt}. The deamidated form of the tryptic peptide (MFDVGGER) is only detectable if $G\alpha_{i2}$ was coexpressed with active PMT-C^{wt}. (*B*) ETD MS/MS spectrum of *m/z* 455.2 (2+) (*Upper*) and of *m/z* 455.7 (2+) (*Lower*) showing a shift of 1 Dalton from z_2 upwards, indicating the deamidation of glutamine-205 to glutamic acid.

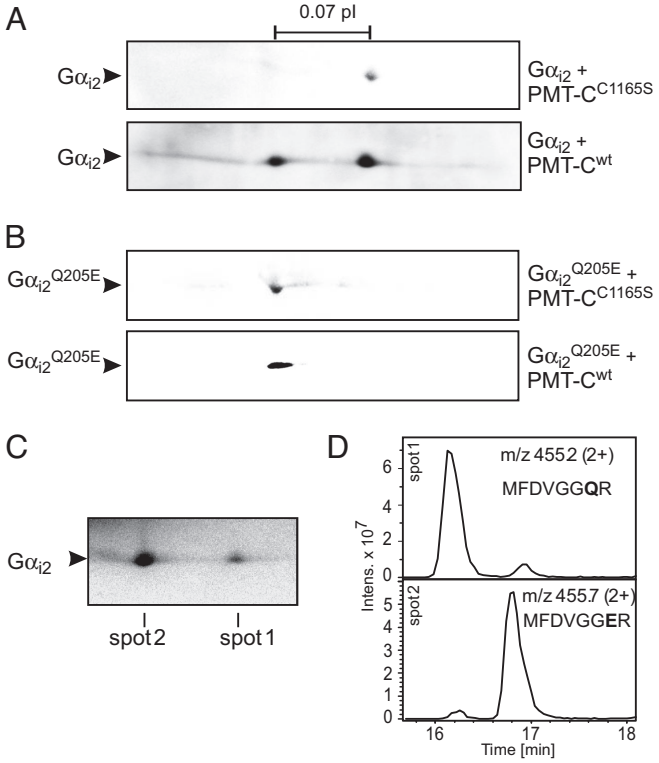

Fig. 3. Change in the isoelectric point (pI) of recombinant $G\alpha_i$ proteins. (*A*) Immunoblot of 2-D electrophoresis gel of $G\alpha_{i2}$ coexpressed with inactive (*Upper*) or active (*Lower*) PMT fragment. $G\alpha_{i2}$ was detected by using anti-$G\alpha_{i1-3}$ antiserum. (*B*) Immunoblot of 2-D electrophoresis gel of $G\alpha_{i2}^{Q205E}$ coexpressed with inactive (*Upper*) or active (*Lower*) PMT fragment. $G\alpha_{i2}$ was detected by using anti-$G\alpha_{i1-3}$ antiserum. (*C*) Coomassie-stained 2-D electrophoresis gel of $G\alpha_{i2}$ coexpressed with active PMT-C^{wt}. (*D*) Spot 1 and 2 were subjected to MS analysis. Extracted ion chromatograms of spot 1 and spot 2 representing amino acids 199–206 of $G\alpha_{i2}$ are shown. Spot 1 results in 1 peak with *m/z* 455.2 (2+) representing unmodified $G\alpha_{i2}$, whereas spot 2 results in a peak with *m/z* 455.7 (2+) representing modified $G\alpha_{i2}$.

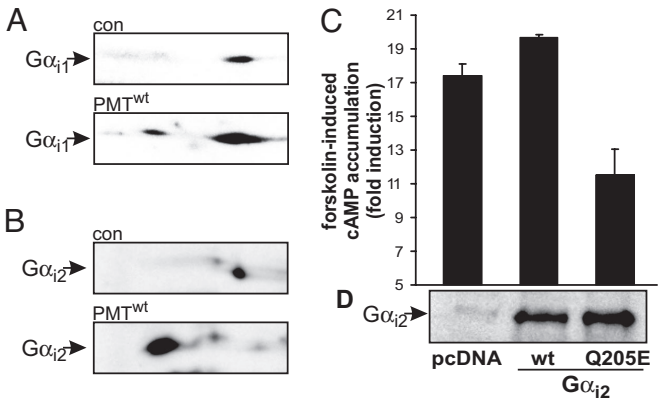

Fig. 4. Activation of $G\alpha_i$ by PMT. (*A* and *B*) MEFs were incubated without (*Upper*) or with PMT^{wt} (10 nM, 16 h, *Lower*), and CHAPS lysates were prepared. Immunoblots of 2-D gel electrophoresis of $G\alpha_{i1}$ (*A*) and $G\alpha_{i2}$ (*B*) are shown. $G\alpha_i$ was detected by using anti-$G\alpha_{i1-3}$ antiserum. (*C*) Inhibition of forskolin-induced cAMP accumulation by $G\alpha_{i2}^{Q205E}$. HEK 293 cells were transfected with empty pcDNA expression vector or with vector encoding $G\alpha_{i2}^{wt}$ or $G\alpha_{i2}^{Q205E}$. Transfected cells were incubated without or with forskolin (40 μM) for 10 min. Shown are the fold induction of cAMP accumulation over buffer control and representative results (mean ± SE, $n = 3$) from at least 3 independent experiments. (*D*) Immunoblot of ectopically expressed $G\alpha_{i2}^{wt}$ and $G\alpha_{i2}^{Q205E}$. Lysates of transfected HEK 293 cells (described in *C*) were prepared, and immunoblot was performed by using $G\alpha_{i1-3}$ antiserum. Endogenous level of expression is observable in lane 1 (empty pcDNA expression vector).

coexpression with PMT-C^{wt}, which was not detected after coexpression with the inactive PMT-C^{C1165S} (Fig. 3*A*). A similar migration shift was detected for the recombinant mutant $G\alpha_{i2}^{Q205E}$. Moreover, coexpression of mutant $G\alpha_{i2}^{Q205E}$ with active or inactive PMT-C in *E. coli* did not further alter the pI of the $G\alpha_{i2}$ protein (Fig. 3*B*). To verify that the pI shift on 2-D gels corresponded with deamidation of $G\alpha_{i2}$, both spots were excised from the gel (Fig. 3*C*), and the trypsin-digested products analyzed by MS. This analysis revealed that the basic protein spot contained the wild-type peptide 199-MFGVGGQR-206, whereas the acidic protein spot corresponded to $G\alpha_{i2}$ deamidated at glutamine-205 (Fig. 3*D*).

PMT-Induced Deamidation Inhibits the GTPase Activity of Recombinant $G\alpha_{i2}$ and Causes a pI Shift of Native $G\alpha_i$ Proteins. It is well known that glutamine-205 of $G\alpha_{i2}$ is essential for GTPase activity. This residue is conserved throughout the GTPase superfamily and is exchanged frequently for leucine to obtain the corresponding constitutively active G proteins (25, 26). To demonstrate that deamidation of glutamine-205 results in inhibition of GTPase activity of $G\alpha_{i2}$, we changed this residue by site-directed mutagenesis to glutamic acid and studied the RGS-stimulated GTPase activity. As expected, the GTPase activity of $G\alpha_{i2}^{Q205E}$ expressed in *E. coli* was inhibited (Fig. S3). Accordingly, over-expression of $G\alpha_{i2}^{Q205E}$ in HEK293 cells caused decreased cAMP accumulation stimulated by forskolin, indicating that G_i signaling was activated by $G\alpha_{i2}^{Q205E}$ (Fig. 4*C*).

All of the experiments thus far were performed with recombinant $G\alpha_i$ proteins. Although we have shown recently that PMT treatment of intact cells inhibits PTx-induced ADP-ribosylation of G_i in cell membranes (16), we wanted to test whether cellular G_i is directly affected by PMT. To this end, MEFs were treated with active full-length PMT^{wt} overnight. The G_i proteins were then isolated from MEF membranes by using CHAPS extraction and analyzed by 2-D gel electrophoresis. $G\alpha_{i1}$ and $G\alpha_{i2}$ were identified by specific antibodies. As shown in Fig. 4, PMT treatment of MEFs caused a shift of $G\alpha_{i1}$ and $G\alpha_{i2}$ migration in the 2-D gel, which was in line with a deamidation of the G proteins, indicating deamidase activity by PMT in intact cells. Notably only a small portion of $G\alpha_{i1}$ was shifted, whereas $G\alpha_{i2}$ was nearly completely modified, suggesting differential substrate specificity of the $G\alpha_i$ isoforms by PMT.

PMT Targets $G\alpha_q$ but Not $G\alpha_{11}$. Next, we wanted to know whether G_q, which is a well-known target of PMT (12) is also deamidated by the toxin. We treated MEFs with PMT^{wt} overnight and, thereafter, obtained CHAPS extracts of the cell membrane fraction. Because 2-D gel analysis was not possible with $G\alpha_q$ from cell membrane extracts, we analyzed the G protein by native gel electrophoresis, which also allows for detection of pI changes of proteins. As shown in Fig. 5*A*, PMT increased the migration of $G\alpha_q$ in native gel electrophoresis, as detected by $G\alpha_q$-specific immunoblot analysis. This effect depended on the time of PMT added to the cell culture (Fig. S4). Notably, under similar conditions PMT did not alter the migration of $G\alpha_{11}$, which was detected by a specific anti-$G\alpha_{11}$ antibody. These findings are in line with previous reports that PMT activates $G\alpha_q$ but not $G\alpha_{11}$ signaling, again verifying the specificity of PMT under these conditions (13, 14). Similar results were obtained from 2D-PAGE analysis of in vitro translated $G\alpha_q$ and $G\alpha_{11}$. Transcription of G_q in the presence of active PMT^{wt} caused a shift of $G\alpha_q$ to acidic pI values. By contrast, no shift of $G\alpha_{11}$ was detectable by PMT-treatment (Fig. S5). All these data supported the view that PMT causes deamidation of $G\alpha_q$ in a similar manner as of $G\alpha_i$.

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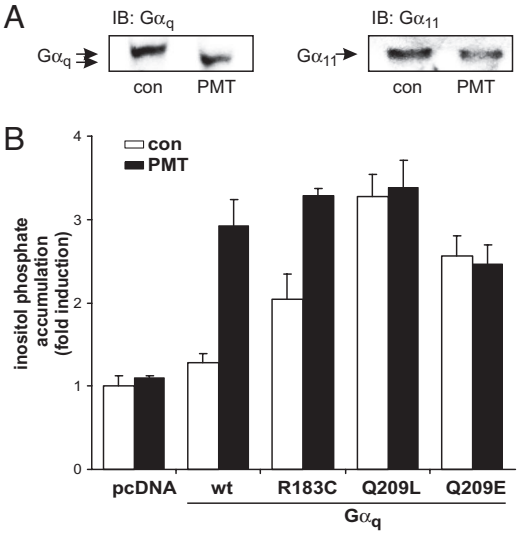

Fig. 5. Activation of $G\alpha_q$ by PMT. (*A*) MEFs were treated without or with PMT (1 nM, 16 h) and membrane extracts were purified. Extracts were subjected to nondenaturating gel electrophoresis and immunoblot was performed with anti-$G\alpha_q$ or anti-$G\alpha_{11}$ antiserum. (*B*) $G\alpha_{q/11}$-deficient MEFs were transfected to express the indicated mutants of $G\alpha_q$ by using the Nucleofection system (Amaxa Biosystems). Cells were stimulated without PMT (white bars) or with PMT (10 nM, black bars) for 4 h. Shown is the fold induction of inositol phosphates above buffer control of cells transfected with empty pcDNA vector. Representative results (mean ± SE, $n = 3$) from at least 3 independent experiments are shown.

We next studied the functional consequences of expression $G\alpha_q$ with mutation at glutamine in position 209 ($G\alpha_q^{Q209E}$) on inositol phosphate formation in $G\alpha_{q/11}$-deficient MEFs and compared the results with expression of wild-type, R183C or Q209L mutant $G\alpha_q$. As expected, PMT^{wt} increased inositol phosphate formation in $G\alpha_{q/11}$-deficient MEFs transfected with wild-type $G\alpha_q$ (Fig. 5*B*), but not $G\alpha_{11}$ (14). Expression of recombinant $G\alpha_q^{Q209E}$ resulted in increased inositol phosphate production. A similar stimulation was observed with the classical Q209L mutant of $G\alpha_q$ (25). Importantly, treatment with PMT did not cause an additional effect on inositol phosphate accumulation. In contrast, PMT^{wt} enhanced inositol phosphate production after expression of the R183C mutant, which is known to be less efficient at inhibiting the GTPase activity of $G\alpha_q$ as the Q209L mutations.

Discussion

PMT is a potent activator of various G proteins including $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$. Here, we elucidated the molecular mechanism of PMT as the deamidation of a specific glutamine residue of $G\alpha$ subunits of G proteins. This conclusion is supported by several findings. First, LC-MS/MS analyses revealed that coexpression of $G\alpha_{i2}$ with an active PMT fragment causes a mass difference of 1 Da representing a deamidation of glutamine-205 to glutamic acid. This deamidation was not observed with inactive PMT, excluding unspecific effects of coexpression. Second, PMT caused a pI shift of recombinant $G\alpha_{i2}$ protein, which is in agreement with deamidation of a glutamine residue. No additional shift was observed after coexpression of PMT with the mutant $G\alpha_{i2}^{Q205E}$, indicating that only a single glutamine residue is modified by deamidation. Third, not only recombinant but also native $G\alpha_i$ proteins are modified by active PMT. Finally, a similar modification induced by PMT was detected with recombinant and native $G\alpha_q$ but not with $G\alpha_{11}$, which is not a substrate of PMT. Furthermore, deamidation of glutamine-205 of $G\alpha_{i2}$ by PMT plausibly explains why PMT prevents the ADP-ribosylation of the G protein by PTx and results in inhibition of basal and RGS-stimulated GTPase activity. Moreover, the deamidase function of PMT is in line with the report of GPCR-independent activation of $G\alpha_q$ by the toxin (27).

So far we were not able to detect a PMT-catalyzed deamidase activity of G proteins in membrane preparations of mammalian cells or with recombinant G proteins, when the toxin was added after preparing the cell membranes or after expression and isolation of the G protein. At least 2 explanations are feasible: First, a defined conformational change of the G protein is necessary for the modification by PMT, which is not achieved in cell membranes or with isolated recombinant G proteins, and second, an additional factor is required for PMT-induced deamidation, which is missing in membrane preparation or with isolated recombinant proteins. Studies are underway to clarify this interesting aspect of the PMT effect.

Glutamine-205 of $G\alpha_{i2}$ (glutamine-204 of $G\alpha_{i1}$) has been described as a key catalytic residue of the inherent GTPase activity of $G\alpha_{i2}$ (28–30) that stabilizes the pentavalent transition state of GTP hydrolyses and helps in orientation of the water nucleophile. Deamidation of this residue to glutamic acid blocks GTP hydrolysis and locks the $G\alpha$ subunit in an active state. It is generally accepted that glutamine-209 of $G\alpha_q$, which is activated by PMT, has the same function in GTP hydrolysis and in regulation of the activity state of G_q. In line with this view, expression of the mutant $G\alpha_q^{Q209E}$ in $G_{q/11}$-deficient MEF caused stimulation of PLCβ-mediated inositol phosphates production, which was not further stimulated by PMT. By contrast, increased formation of inositol phosphates production observed in the R183C mutant of $G\alpha_q$ was further stimulated by PMT. An amino acid residue equivalent to glutamine-205 of $G\alpha_{i2}$ and glutamine-209 of $G\alpha_q$ is also present in $G_{12/13}$ proteins. Therefore, we propose that PMT activates $G\alpha_{12/13}$ also by deamidation of this residue. Glutamine-209 is also present in G_{11}, which is not activated by PMT. So far we do not know the reason why G_{11} is not activated by PMT. However, it was recently shown that the αB-helix located in the helical domain of $G\alpha_{11}$ is essential for prevention of the PMT effect (14). Therefore, we speculate that interaction of PMT with $G\alpha_{11}$ is functionally restricted by structural features of G_{11}, at least part of which are located in the helical domain.

Functionally equivalent to glutamine-205 of $G\alpha_{i2}$ is glutamine-63 in the small GTPase RhoA. RhoA is activated by *E. coli* cytotoxic necrotizing factors (CNFs) by deamidation of glutamine-63 (31, 32). Interestingly, PMT shares similarity with CNFs in the N-terminal binding and translocation domains. However, the C-terminal catalytic domain of CNFs has no obvious structural similarity with PMT, as evidenced by comparison of the crystal structure of the C-terminal C3 domain of PMT (19), harboring the minimal intracellular activity domain (20), with that of the catalytic domain of CNF1 (33) (Fig. S6 *A* and *B*). PMT and CNF1 share the catalytic residues histidine and cysteine, whereas the third catalytic residue is aspartate in the case of PMT and valine in CNF1. The topography of the catalytic center of PMT with its key residues cysteine-1165, histidine-1205 and aspartate-1220 is different from that of CNF1 with cysteine-866, histidine-881 and valine-833 (Fig. S6*C*). It has been shown that the positioning of the catalytic triad of PMT is very similar to the catalytic triad of papain and mainly deduced from this similarity PMT has been proposed to act as a protease (19). Our findings indicate that PMT acts as a deamidase to activate G proteins. Structural comparison of deamidases and thiol proteases with transglutaminases explains this discrepancy and shows that previous data on the crystal structure of PMT are not contradictory to our findings. From the type of chemical reaction, transglutaminases, which replace the NH_2-group of the amide of glutamine by another amine residue, are closely related to deamidases, which change the amide to a carboxylate. Transglutaminases possess a

similar catalytic triad as PMT and papain proteases (33–35). For example, the catalytic triad (Cys-His-Asp) of the transglutaminases factor XIII perfectly matches the position of the catalytic triad of PMT (Fig. S6*D*).

The potent mitogenic activity of PMT observed in some cell types has stimulated discussions on the role of the toxin in tumor development and carcinogenesis (10, 36). Interestingly, mutations of the α-subunits of G protein, which cause inhibition of intrinsic GTPase activity and formation of constitutively active forms of the G protein have been observed in certain types of tumors and/or have been implied in tumorigenesis. For example, $G\alpha_{i2}$ mutation at position 205 has been observed in pituitary adenomas (37). Quite early the Q209L mutant gene of $G\alpha_q$ was shown to act as a fully transforming oncogene (38). More recently, van Raamsdonk and coworkers reported that frequent somatic mutations in the gene *Gnaq*, encoding $G\alpha_q$, are observed in melanoma of the uvea (46%) and in blue naevi (83%). The mutations occur exclusively in codon 209, resulting in similar constitutive activation as reported here for PMT (39). In addition, constitutively activated mutants of $G\alpha_{12/13}$ have been shown to be potent in cellular transformation (40) and activation of $G\alpha_{13}$ may have a crucial role in tumor invasion and migration (41). Therefore, it is feasible that PMT-induced activation of G proteins by deamidation plays a role in cancer development.

Taken together our data show that PMT acts as a deamidase to activate the α-subunits of a specific subset of heterotrimeric G proteins and explains the activation of various signaling pathways by the toxin.

Methods

Plasmid Vector Construction. The cDNA clones for human $G\alpha_{i2}$ and $G\alpha_q$ in pcDNA3.1 were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). $G\beta_1$ and $G\gamma_2$ in pcDNA3.1 were a kind gift of Dr. B. Nürnberg (Universität Tübingen, Tübingen, Germany). RGS3s and RGS16 were a kind gift of Dr. T. Wieland (Universität Heidelberg, Heidelberg, Germany). Further details of cloning and mutations are provided in *SI Materials and Methods* and Table S1.

Protein Expression. PMT, PMT fragment (PMT-C) harboring the biological active domain, RGS3 and RGS16 were purified as described in refs. 18 and 42. For details of coexpression of inactive PMT-C^{C1165S} or active PMT-C^{wt} with $G\alpha_{i2}$ see *SI Materials and Methods*.

LC-MS/MS Analysis. Prepared peptides were subjected to LC-MS/MS analyses on an ion trap mass spectrometer (Agilent 6340, Agilent Technologies) equipped with an ETD source and coupled to an 1200 Agilent nanoflow system via a HPLC-Chip cube ESI interface (see details in *SI Materials and Methods*).

Cell Culture. Mouse embryonic fibroblasts (MEFs) derived from $G\alpha_{q/11}$-deficient or wild-type mice were cultured as described in ref. 43. For details of transfection see *SI Materials and Methods*.

Detection of $G\alpha_i$ by PTx-Induced ADP-Ribosylation and Immunoblot Analysis. PTx-induced ADP-ribosylation of $G\alpha_i$ was carried out as described in ref. 44. $G\beta_1\gamma_2$ complex was a kind gift of Dr. B. Nürnberg. For Western-blotting, samples were subjected to SDS/PAGE and transferred onto polyvinylidene difluoride-membrane. $G\alpha_i$ was detected by using anti-$G\alpha_{i1-3}$ serum, which was a kind gift of Dr. B. Nürnberg. $G\alpha_q$ and $G\alpha_{11}$ were detected by using specific antibodies (Santa Cruz Biotechnology). Immunoblots were visualized by using the LAS-3000 imaging system (Fujifilm).

Two-Dimensional Polyacrylamide Gel Electrophorsis (2-D-PAGE). Isoelectric focusing was performed on an IPGphor device (Amersham) by using an 18 cm linear gradient of pH 5–6 ready-to-use Immobiline DryStrips (GE Healthcare). The 2nd dimension was performed by conventional SDS/PAGE. For preparation of membrane extracts see *SI Materials and Methods*.

G$\beta\gamma$ Pull-Down Assay. Precipitation of in vitro translated G$\beta\gamma$ with immobilized GST-$G\alpha_{i2}$ was performed as described in ref. 45. For details see *SI Materials and Methods*.

Single Cycle GTPase Activity. Intrinsic GTPase activity of recombinant $G\alpha_{i2}$ was measured as described in ref. 46.

cAMP Accumulation Assay. cAMP was measured by the double column technique by using Dowex AG50-X8/alumina columns. For details see *SI Materials and Methods*.

Analysis of Total Inositol Phosphates. Analysis of total inositol phosphates was performed as described in ref. 18.

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