His1205 and His1223 Are Essential for the Activity of the Mitogenic *Pasteurella multocida* Toxin[†]

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ABSTRACT: *Pasteurella multocida* produces a 146-kDa protein toxin (PMT), which activates multiple cellular signal-transduction pathways, resulting in the activation of PLCβ, Rho, JNK, and ERK. In addition to an essential cysteine residue at position 1165, PMT contains several histidine residues in the catalytically important C-terminal part of the protein. To elucidate the role of the histidine residues, we treated PMT with the histidine-modifying substance diethyl pyrocarbonate (DEPC). DEPC inhibited PMT in a time- and concentration-dependent manner, suggesting that one or several histidine residues are essential for the biological activity of PMT. In experiments in which PMT was directly delivered into the cytosol of EBL cells by electroporation, we show that DEPC treatment inhibits the catalytically important histidine residues. Leucine substitutions of eight individual histidine residues in the C-terminal catalytic domain of PMT were constructed, and the effect on the biological activity of PMT was analyzed by determining PLCβ, Rho, and ERK activation. Substitution of two histidine residues, H1205 and H1223, led to inactivation of the resulting PMT proteins, indicating that H1205 and H1223 play an important role in biological activity of the toxin. In addition, we show that the mutant toxins appear to be correctly folded, as judged by protease digestion. The precise function of H1205 and H1223 is not yet known. However, treatment of PMT with the cation chelating substance 1,10-phenantroline led to inactivation of the toxin, indicating that the essential histidine residues and cysteine 1165 might be involved in metal ion binding.

Pasteurella multocida induces localized inflammation, pneumoniae, bacteremia, and atrophic rhinits in pigs (*1*). The Gram negative bacterium produces a 146-kDa protein, *Pasteurella multocida* toxin (PMT),[1] which appears to be the causative agent of atrophic rhinitis (*1*). The toxin binds to a ganglioside-type cell surface receptor and has to be internalized via receptor-mediated endocytosis to elicit its cytotoxic effects (*2*−*4*). PMT activates numerous cellular signal transduction pathways. In contrast to natural inducers of signaling pathways, PMT leads to a persistent activation. The toxin is a strong mitogen, leading to increased DNA synthesis and proliferation in several cell lines (*2*, *3*, *5*). The mitogenic action depends on the stimulation of the MAP-kinase ERK (*6*). PMT stimulates G_q-dependent phospholipase Cβ1 (PLC-β1) (*7*), resulting in the mobilization of calcium, accumulation of diacylglycerol, and activation of protein kinase C (*8*). Recently, it was shown in cell lines deficient in G_q, G_{11}, or both that stimulation of PLCβ1 is mediated by G_q, but not by the closely related G_{11} (*9*). In addition, the small GTPase Rho is activated by PMT, resulting in the formation of stress fibers and focal adhesions and tyrosine phosphorylation of focal adhesion kinase and paxillin (*3*, *10*). Interestingly, the activation of Rho, ERK, and JNK is independent of G_q, showing that PMT activates signaling pathways in a G_q-dependent and -independent manner (*9*). However, the precise target and mode of molecular action of PMT is not yet known.

PMT consists of 1285 amino acid residues. Initial studies revealed that the N-terminus contains the binding and translocation domain (*11*), whereas the catalytic domain is located in the C-terminal part of the protein (*11*, *12*). In the C terminus, an essential cysteine residue was identified (*13*). It was suggested that this cysteine plays a role in the biological activity by forming metal ion complexes (*13*).

Here we report that DEPC, which specifically modifies histidine residues, inactivates PMT. We have identified two residues, H1205 and H1223, that are essential for the intracellular activity of PMT.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]-labeled inositol was obtained from DuPont NEN (Dreieich, Germany). PCR primers were from MWG Biotech (Ebersberg, Germany). The pGEX-2T vector and the glutathione-*S*-transferase gene fusion system were from

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[1] Abbreviations: ATP, adenosine-5-triphosphate; DEPC, diethyl pyrocarbonate; EBL cells, embryonic bovine lung cells; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GST, glutathione-*S*-transferase; IB, immunoblot; IP, inositol phosphate; JNK, c-Jun terminal kinase; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PLCβ, phospholipase Cβ; PMT, *Pasteurella multocida* toxin; PTL, phenanthroline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylendiamine.

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Amersham Bioscience (Uppsala, Sweden). The Quick-Change kit was from Stratagene (Heidelberg, Germany). 1,10-Phenanthroline was from Calbiochem-Novabiochem (Schwalbach, Germany). 1,7-Phenanthroline and diethyl pyrocarbonate were from Sigma-Aldrich (Taufkirchen, Germany). All other reagents were of analytical grade and were purchased from commercial sources.

Treatment of PMT with DEPC. DEPC was used as described previously (*14*). A stock solution of DEPC, dissolved in ethanol, was added to PMT (100 ng/μL) to a final concentration of 0.5 to 2.0 mM. Ethanol was used as a control. The reaction was allowed to proceed for the indicated times at 30 °C and was stopped by addition of imidazole (final concentration 10 mM).

Treatment of PMT with Phenanthroline. PMT (1 μg) was diluted in 30 μL of buffer, containing Tris (50 mM, pH 8), NaCl (50 mM), and $CaCl_2$ (2.5 mM), and was incubated with 20 mM 1,10-phenanthroline or 1,7-phenanthroline for 30 min at 37 °C. Chelator-free buffer was used as a control. The samples were stored on ice until toxin activity was assayed.

Construction of Mutant PMT. Mutated proteins were constructed by site-directed mutagenesis using pGEX-2T-wt-PMT plasmid as template and the respective oligonucleotides using the Quick-Change kit according to the manufacturer's instructions. The nucleotide changes responsible for mutation are underlined. From the two complementary primers used for each mutation, only one is listed: H622L, 5′-CTA CAA AGA ATA CTT AAC AGT AAT ATC-3′; H635L, 5′-CAA GGT TTA ATG CTT GAA CTC ATG GAG-3′; H635A, 5′-CAA GGT TTA ATG GCT GAA CTC ATG GAG-3′; H804L, 5′-CTT TAT ATT GGA CTT AGC TAT GAA GAA-3′; H1001L, 5′-AGA GCT ATA GGG CTT TCA GAT AAT TCT-3′; H1202L, 5′-ATG GAA TTT TCA CTT CAG ATG CAT ACT-3′; H1202A, 5′-ATG GAA TTT TCA GCT CAG ATG CAT ACT-3′; H1205L, 5′-TCA CAT CAG ATG CTT ACT ACT GCT TCC-3′; H1223L, 5′-GTG GAT GCT TCA CTT TTA CAA TTT GTA-3′; H1228L, 5′-TTA CAA TTT GTA CTT GAC CAA TTA GAT-3′. The plasmids were transformed in *Escherichia coli* TG1 cells. All mutations were confirmed by DNA sequencing with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Überlingen, Germany).

Expression and Purification of PMT Proteins. Recombinant PMT proteins were expressed as glutathione-*S*-transferase (GST) fusion proteins and purified according to the manufacturer's instructions (Amersham Pharmacia Biotech). In brief, GST-fusion proteins were isolated by affinity chromatography with glutathione-Sepharose, followed by proteolytic cleavage using 3.25 U thrombin/mg of recombinant GST-fusion protein. Thrombin was removed by incubation with benzamidine-Sepharose (Amersham Pharmacia Biotech).

Analysis of Total Inositol Phosphates. EBL cells were grown in 24-well plates for 2 days and labeled with 2 μCi/mL of [2-^{3}H]-inositol in serum-free medium (MEM) for 12 h. Subsequently, PMT or PMT fragments and LiCl (20 mM) were added and the cells were incubated for the indicated times. For cell lysis and extraction of inositol phosphates, the medium was replaced with 750 μL ice-cold formic acid (10 mM, pH 3). After 30 min incubation on ice, the extract was neutralized with 3 mL of NH_3 (5 mM, pH 8.5). Analysis of total inositol phosphates was done by anion exchange chromatography using AG1-X8 resin (200 to 400 mesh; Bio-Rad, München, Germany) as described previously (*12*).

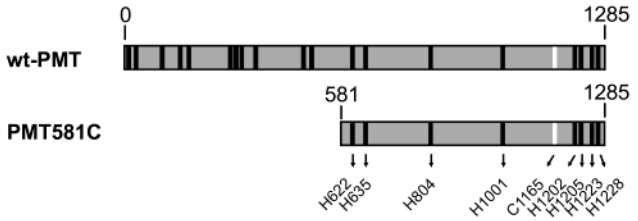

FIGURE 1: Schematic representation of wt-PMT and PMT581C. The black bars indicate the histidine residues. The white bar indicates the essential cysteine residue.

Electroporation of EBL cells. Confluent EBL cells were harvested 16 h after labeling with 2 μCi/mL [2-^{3}H]-inositol. A mixture of the PMT proteins or substances and 5×10^5 cells in 400 μL of MEM + 15% FCS were electroporated in a 0.4-cm gap-width electroporation cuvette (Bio-Rad, Munich, Germany). Electroporation was performed using a Gene Pulser Transfection Apparatus (Bio-Rad) using a capacity of 950 μF and a voltage of 200 V. After electroporation, 100 μL aliquots of the cell suspension were transferred to 24-well plates, containing 500 μL of prewarmed MEM + 15% FCS, and were incubated for the indicated times at 37 °C and 5% CO_2. The amount of total inositol phosphates was determined as described above.

Binding/Uptake Competition Experiments. EBL cells were preincubated with 500-fold excess of DEPC-treated or mutated PMT preparation for 15 min at 37 °C. Then 100 ng/mL wt-PMT was added to the cells. After 15 min, the medium was aspirated, fresh medium, containing 20 mM LiCl was added and IP-accumulation was analyzed as described above.

Proteolytic Digestion of PMT. PMT and various PMT mutants (5 μg) were digested with trypsin (50 ng of trypsin/5 μg of PMT) at 37 °C for 20 min. The reaction was stopped by adding Laemmli sample buffer to the samples, and SDS−PAGE was performed as described by Laemmli (*15*).

Determination of ERK Phosphorylation. Mouse fibroblast cells were seeded in six-well plates (2 mL of DMEM + 15% FCS) and serum starved for 24 h. The cells were incubated with PMT proteins (100 ng/mL, 3 h) and EGF (10 ng/mL, 5 min) at 37 °C. The cells were washed once with ice-cold PBS and lysed with 30 μL/well Laemmli sample buffer. Cell lysates were separated by SDS−PAGE, and phosphorylation was determined by subsequent immunoblotting with a phosphospecific anti-ERK antibody (Santa Cruz Biotechnology). To confirm equal loading of ERK1/2, membranes were stripped and reprobed with an anti-ERK antibody (Santa Cruz Biotechnology).

Activation of Rho in NG108−15 cells. For determination of Rho activation, serum-starved NG108−15 cells grown in six-well plates (24 h, neurobasal medium containing 2% supplement B27) were incubated with PMT-proteins (250 ng/mL, 37 °C). Phase-contrast pictures of the cells were taken after a 3-h incubation and retraction of neurites was determined.

RESULTS

Inactivation of PMT with Diethyl Pyrocarbonate (DEPC). PMT contains several histidine residues in close proximity to the catalytically essential cysteine 1165 (Figure 1).

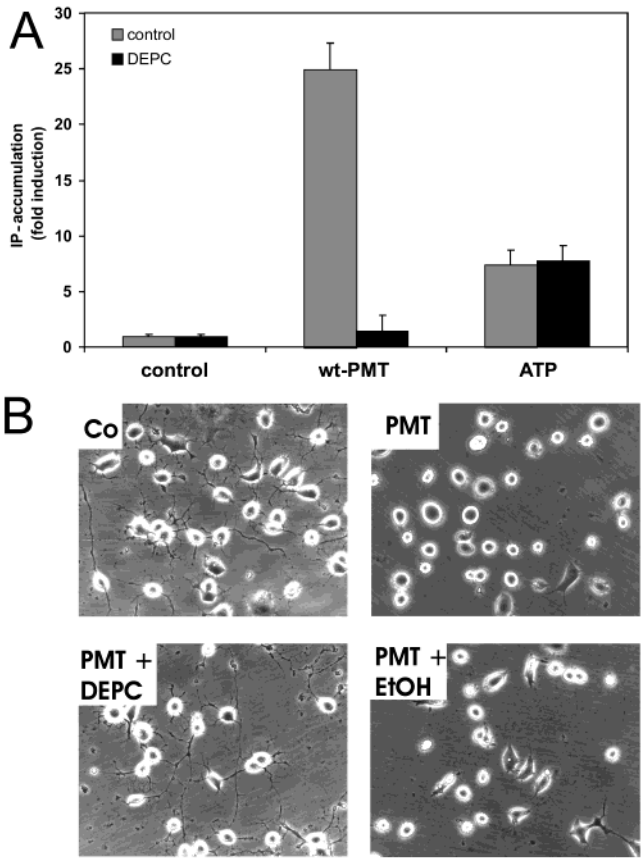

FIGURE 2: Inactivation of PMT by diethyl pyrocarbonate. (A) wt-PMT was treated with 2 mM DEPC and added to [^{3}H]-inositol-labeled EBL cells. After 3 h, the total amount of inositol phosphates in the cells was measured. As a control, the purine receptor agonist ATP was added at the same time to the medium. wt-PMT was applied at a concentration of 1 μg/mL, and ATP applied at 1 mM. Data are given as fold induction over buffer control (means $\pm$ standard error; $n = 3$). Representative results of at least three independent experiments are shown. (B) Pretreatment of wt-PMT with DEPC prevents the retraction of neurites in NG 108−15 cells. NG 108−15 cells were incubated for 24 h in serum-free neurobasal medium plus supplement B27. The cells were incubated with 100 ng/mL wt-PMT or DEPC-treated wt-PMT. After 2 h, photographs were taken. As a control, wt-PMT was treated with ethanol.

Because a number of enzymes contain catalytically important histidines, we were interested in determining whether any of these histidine residues are important for the catalytic activity of PMT. wt-PMT was treated with the histidine-modifying substance DEPC, and the toxicity of the DEPC-treated PMT was assessed by analyzing two different signaling pathways that are stimulated by PMT. First we studied PMT-induced PLC-β1 activation in EBL cells by measuring the accumulation of inositol phosphates. Addition of wt-PMT to the [^{3}H]inositol-labeled monolayers induced a 25-fold increase in inositol phosphate (IP) accumulation (Figure 2A), indicating PLC-β1 activation. Pretreatment of PMT with DEPC abolished the PMT-dependent activation of PLC. To show that DEPC itself had no effect on the activity of PLC, we stimulated a G_q-coupled purine-receptor with ATP in the presence of DEPC. Addition of ATP resulted in a 8-fold increase of IP accumulation, which was not inhibited by DEPC (Figure 2A).

To further characterize the effect of DEPC treatment, we examined the ability of PMT to induce Rho activation after DEPC treatment. Studies with the neuronal cell line NG108−15 have shown that activation of the small GTPase Rho leads to a retraction of neurites (*16*). Rho activation can be easily monitored in NG108−15 cells by using phase contrast microscopy, thus providing a useful model to study PMT-mediated Rho activation. To allow neurite outgrowth, NG108−15 cells were kept overnight in serum-free neurobasal medium containing supplement B27. PMT was then added and retraction of neurites was monitored. As shown in Figure 2B, addition of wt-PMT to NG 108−15 cells led to retraction of neurites within 2 h. Pretreatment of wt-PMT with DEPC inhibited this effect. As a control, wt-PMT was treated with an equivalent amount of ethanol to that used to solubilize DEPC. Incubation of PMT with ethanol had no effect on PMT activity. These results indicated that one or several histidine residues are essential for the biological activity of PMT.

DEPC Inhibits Activity of the Uptake Insufficient PMT581C. The N-terminus of PMT, which is important for the uptake of the toxin into cells also contains several histidine residues (Figure 1). To test whether the loss of toxicity after preincubation of PMT with DEPC was due to an inhibition of toxin binding or uptake into the cells, we analyzed the activity of DEPC-treated PMT after introducing the toxin by electroporation into cells. To exclude toxin uptake by its normal route, we used a C-terminal fragment of PMT (PMT581C), which does not harbor the receptor binding and translocation domain and which elicits its biological activity only after bypassing the uptake mechanism (*12*). PMT581C was treated with DEPC and its PLC-stimulating activity was tested after electroporation into EBL cells. Similar to wt-PMT, PMT581C was completely inactivated by DEPC (Figure 3B). These results indicate that the modification of catalytically important histidine residues results in inactivation of the toxin.

DEPC Does Not Affect PMT Binding. To address whether DEPC also affects binding/uptake of PMT, we performed competition experiments (Figure 6). EBL cells were preincubated with 500-fold excess of DEPC-treated PMT for 15 min. Then wt-PMT was added. After further 15 min, the medium was replaced and PLC-stimulating activity was measured after 3 h incubation. wt-PMT alone induced 8-fold increase in IP accumulation. Prebinding of DEPC-treated PMT to EBL cells reduced IP accumulation to 3-fold increase. Protection of EBL cells against wt-PMT by DEPC-treated PMT suggest that DEPC-treated PMT still binds to its receptor.

Time and Concentration Dependency of PMT Inhibition by DEPC. We next studied the time and concentration dependency of DEPC-dependent inhibition of PMT. wt-PMT was incubated for various time intervals with increasing concentrations of DEPC. As shown in Figure 3, a concentration of 2 mM DEPC reduced the PLC-stimulating activity of wt-PMT to 20% within 5 min, and resulted in complete inhibition after 10 min incubation. Similarly, PMT581C was almost completely inactivated after incubation with 2 mM DEPC for 10 min. DEPC-dependent inactivation of the C-terminal fragment was faster than that occurring with wt-PMT. A 50% reduction of toxin activity at a concentration of 0.5 mM DEPC was obtained after 4 min for PMT581C and after 10 min for wt-PMT. The results indicate that DEPC inactivates wt-PMT and PMT581C in a concentration- and time-dependent manner.

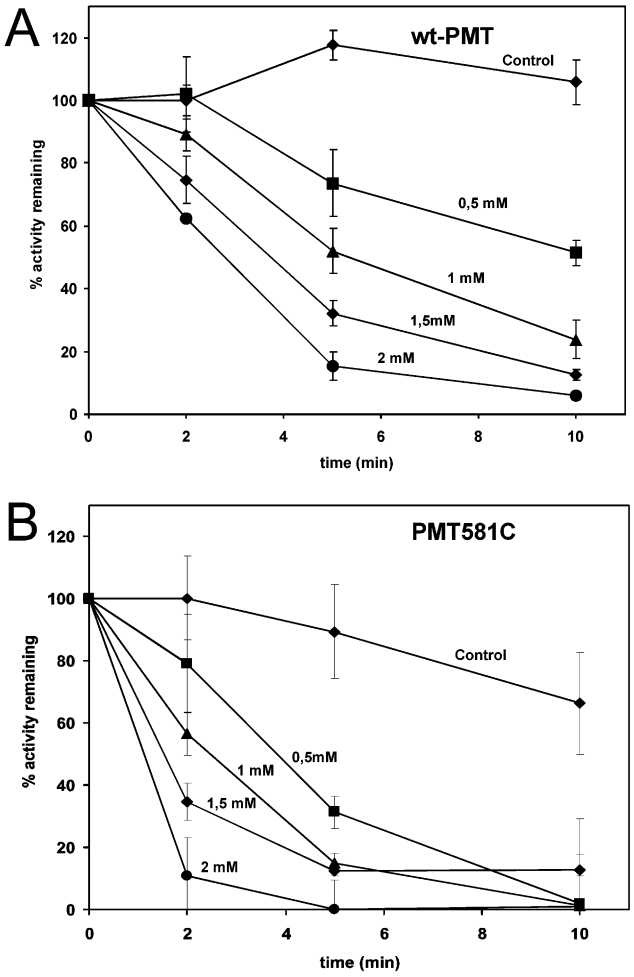

FIGURE 3: Time-dependent inactivation of wt-PMT (A) and PMT581C (B). The toxin was exposed to the indicated concentrations of DEPC and activity of PLC was monitored, determined by an IP-accumulation assay, as a function of exposure time. wt-PMT and PMT581C were added to the culture medium or electroporated into EBL cells, respectively. The activity of wt-PMT and PMT581C was determined by measuring PLC activity.

Mutation of Histidine Residues. The fact that DEPC inhibited PMT suggested that one or more histidine residues are essential for the biological activity of PMT. The schematic overview of wt-PMT and PMT581C in Figure 1 shows that eight histidine residues are located in the catalytically important C-terminal part of the protein (residues 581−1285). To study the role of the histidine residues, they were individually replaced by leucine residues by site-directed mutagenesis. The mutated proteins were expressed as GST-fusion proteins in *E. coli* and purified by affinity chromatography as described in Experimental Procedures. Attempts to purify the proteins PMT.H635L and PMT.H1202L were not successful because of instability of these mutated proteins. Therefore, we mutated H635 and H1202 to alanines. The resulting proteins, PMT.H635A and PMT.H1202A, were well expressed and could be purified (Figure 4A).

The stability of the mutant PMT proteins was studied by using tryptic digestion. Wild type and mutant PMT proteins were incubated with 10 ng of trypsin per μg of PMT for 20 min and then separated by SDS−PAGE (Figure 4B). The mutated proteins exhibited protease digestion profiles similar to wt-PMT, indicating that the point mutations did not grossly affect the folding of the proteins.

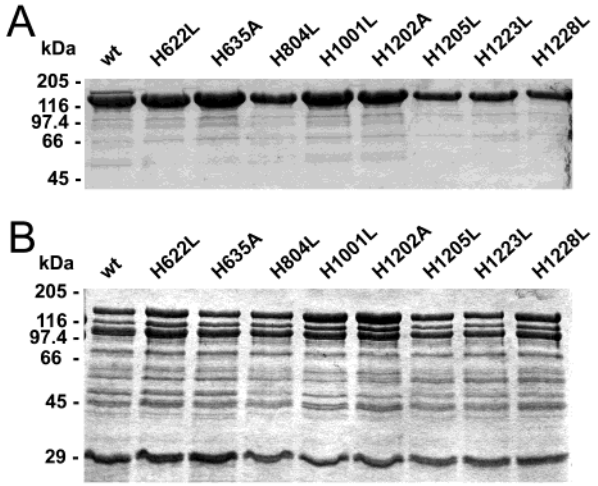

FIGURE 4: SDS−PAGE comparison of wt-PMT and PMT mutated proteins. (A) wt-PMT and PMT mutants were constructed as GST-fusion proteins, expressed in *E. coli*, purified by affinity chromatography, and cleaved by treatment with thrombin. A total of 5 μg of wt-PMT or mutant PMT was loaded on the lanes. For control, we expressed and purified protein from an empty pGEX-2T plasmid. As expected, no protein appears on SDS−PAGE after thrombin cleavage (data not shown). (B) SDS−PAGE comparison of trypsin-treated wt-PMT and mutant PMT. Wt-PMT and the mutants were incubated with 50 ng of trypsin per 5 μg of PMT for 20 min and separated by SDS−PAGE.

Biological Activity of Mutant PMT. PMT-mediated PLC activation was analyzed by measuring IP accumulation in [^{3}H]-inositol-labeled EBL cell monolayers. Mutants H622L, H635A, H804L, H1001L, and H1202A showed similar PLC activation as wt-PMT (30-fold increase in IP-accumulation, Figure 5). The mutant PMT.H1228L activated PLC to a lesser extent (10-fold increase in IP-accumulation). Two PMT mutants, H1205L and H1223L, and the cysteine mutant C1165S had no stimulating effect on PLC activity (Figure 5).

The same results were obtained when the ability of the histidine mutants to induce neurite retraction in NG108−15 cells was examined (Table 1). Addition of PMT.H1205L and PMT.H1223L to the cells did not induce any morphological changes after several hours of incubation, even at high toxin concentrations. PMT.H1228L induced neurite retraction only upon prolonged incubation or with 2−3-fold increased toxin-concentration.

Next we studied the mitogenic effect induced by wt-PMT and mutated proteins by measuring the phosphorylation of ERK 1/2 in mouse fibroblast cells by Western blot analysis. wt-PMT and all but two of the mutant toxins induced phosphorylation of ERK 1/2 (Figure 5B). In contrast, PMT mutants H1205L and H1223L showed no activity, even at very high concentrations (100 μg/mL). For control, ERK 1/2 was stimulated by adding EGF to the cells. To confirm the slight increase in ERK 1/2 phosphorylation, which is induced by PMT.H1228L, we studied the concentration dependency of the effect (Figure 5C). We compared the activity of PMT.H1228L with wt-PMT. wt-PMT induced ERK 1/2 activation at concentrations of 5−10 ng/mL. The mutant PMT.H1228L had no effect until a concentration of 100−500 ng/mL.

The mutant H1205L was also tested for its ability to compete with wt-PMT in a binding/activity assay. As found

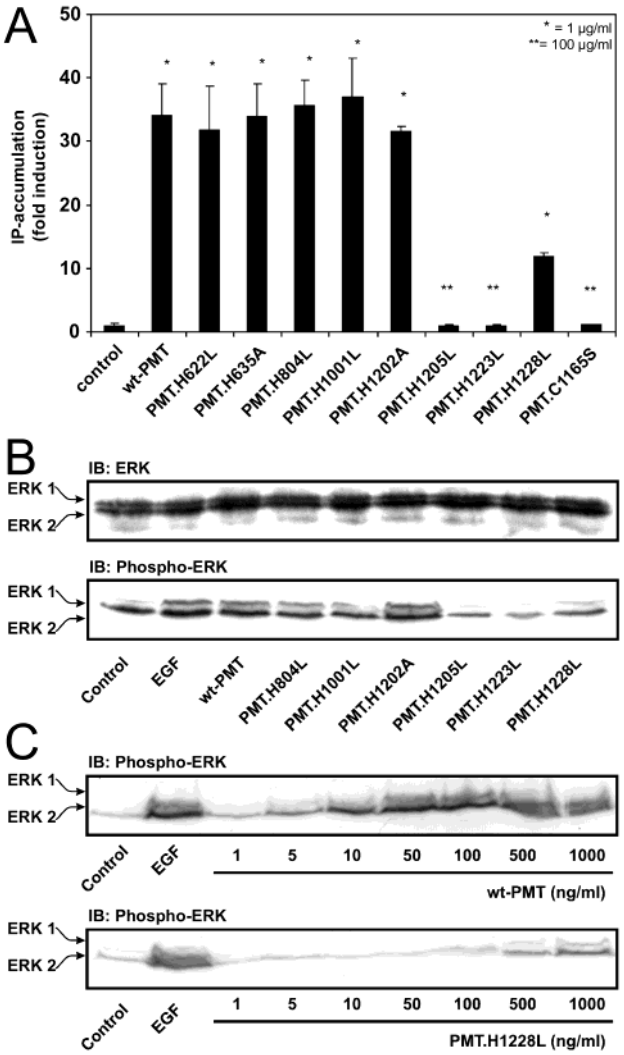

FIGURE 5: Activity of PMT histidine mutants. (A) Effect of PMT mutants on inositol phosphate accumulation in EBL cells. The toxins were applied to EBL cells. After 2 h, the total amounts of inositol phosphates were measured. Data are given as fold induction of buffer control (means and standard error; $n = 3$). Proteins were applied at 1 μg/mL (wt-PMT, PMT.H622L, PMT.H635A, PMT.H804L, PMT.H1001L, PMT.H1202A, PMT.H1228L) or 100 μg/mL (PMT.H1205L, PMT.1223L, PMT.C1165S). (B) Effects of PMT mutants on ERK 1/2 phosphorylation. Serum-starved mouse fibroblasts were incubated with PMT mutants for 3 h or EGF for 5 min. The cells were then lysed and activation of ERK was determined by immunoblotting (IB) with an anti-phospho-ERK antibody. As a control, the same blot was reprobed with an anti-ERK antibody. A representative blot of three independent experiments is shown. (C) Concentration dependency of wt-PMT and PMT.H1228L on ERK 1/2 phosphorylation. Serum-starved mouse fibroblasts were incubated with increasing concentrations of wt-PMT or PMT.H1228L for 3 h or EGF for 5 min. ERK 1/2 phosphorylation was determined as described above. Same results were obtained in two independent experiments.

for DEPC-treated PMT, PMT.H1205L significantly competed with wt-PMT, suggesting no major changes in the overall structure of the protein (Figure 6).

PMT581C Inhibition by 1,10-Phenanthroline. To determine whether binding of metal ions is important for the biological activity of PMT, we treated the toxin with chelating substances. 1,10-Phenanthroline is a chelator of cations commonly used to inactivate enzymes by binding cations at their active centers (*17*−*21*). PMT581C was treated with 20 mM 1,10-phenanthroline at 37 °C for 30 min. The toxin was then electroporated into EBL cells and IP accumulation was measured. As shown in Figure 7A, 1,10-phenanthroline inactivates PMT581C. As a control, cells were treated with the purine-receptor agonist ATP in the presence of 1,10-phenantroline. To exclude any unspecific effect of 1,10-phenanthroline, we compared the inhibition of PMT581C induced by the chelating agent 1,10-phenanthroline and the nonchelating isomer 1,7-phenanthroline. Only the chelating agent was able to inhibit the activity of PMT581C, indicating that the depletion of cations led to inactivation of PMT (Figure 7B).

Table 1: Neurite Retraction by PMT Mutants in NG108−15 Cells

clone	retraction[a]
wt-PMT	+++
H622L	+++
H635A	+++
H804L	+++
H1001L	+++
H1202A	+++
H1205L	-
H1228L	-
H1228L	+

[a] The retraction of neurites was determined after treatment of NG 108−15 cells for 3 h with 250 ng/mL protein. +++, retraction of neurites; +, delayed retraction of neuritis; −, no retraction of neurites.

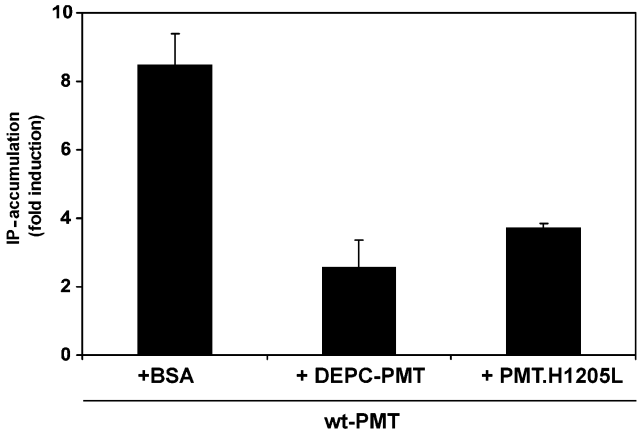

FIGURE 6: DEPC-treated PMT and PMT.H1205L protects EBL cells against wt-PMT activity. EBL cells were preincubated with DEPC-treated PMT, PMT.H1205L, or BSA in a 500-fold molar excess for 15 min at 37 °C. wt-PMT was added at a concentration of 100 ng/mL for 15 min at 37 °C. Then the medium was aspirated and fresh medium, containing 20 mM LiCl was added. After 4 h, total amounts of inositol phosphates were measured. Data are given as fold induction over buffer control (means ± standard error; $n = 3$). Same results were obtained in two independent experiments.

DISCUSSION

In a previous study, it was shown that the most C-terminal cysteine residue is essential for the biological activity of PMT (*13*). It was suggested that this cysteine residue could be involved in membrane interaction or in the catalytic action of PMT. Recently, we studied several fragments of PMT (*12*) and found that the fragment PMT581C, which contains the essential C-terminal cysteine residue, harbors the biological activity. When we analyzed the amino acid sequence of this putative catalytic domain, we found several histidine residues near the essential cysteine residue. This cluster of histidines also attracted the attention of Petersen (*22*). To

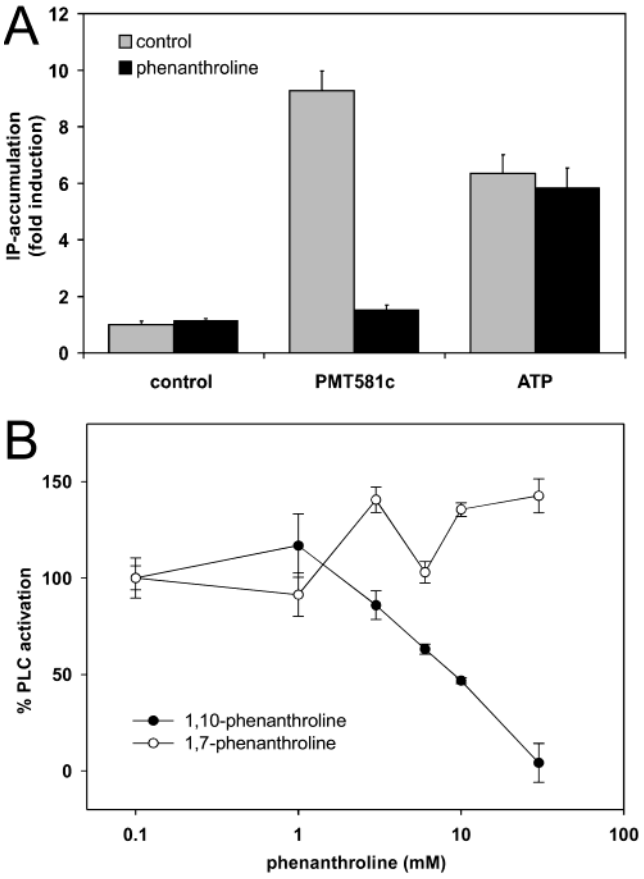

FIGURE 7: 1,10-Phenanthroline inactivates PMT581C. (A) PMT581C was treated with 20 mM 1,10-phenanthroline. The protein was introduced into EBL cells by electroporation. For control, cells were electroporated in the presence of buffer containing 20 mM 1,10-phenanthroline. After 3 h, the total amount of inositol phosphates in the cells were measured. The purine receptor agonist ATP was added to the medium 2 h after electroporation. Data are given as fold induction over buffer control (means ± standard error; $n = 3$). PMT581C was applied at a concentration of 1 μg/mL, and ATP at 1 mM. (B) PMT581C was exposed to the indicated concentration of 1,10-phenanthroline or 1,7-phenanthroline. The percent remaining activity is shown, with the activity of untreated protein set as 100%.

study the role of the histidine residues in PMT, we used diethyl pyrocarbonate, which binds to histidine residues and thereby blocks histidine-based enzyme activity (*14*, *23*, *24*). We found that DEPC blocks wt-PMT and PMT581C induced PLC activation and additionally inhibited wt-PMT induced Rho activation. The inhibition was time- and concentration-dependent. Because DEPC inhibited PMT581C, which was electroporated into cells, we could exclude that DEPC affects only the uptake mechanism of the toxin, e.g., receptor binding or translocation. To test the receptor binding-activity of DEPC-treated PMT, we performed competition experiments between wt-PMT and DEPC-treated PMT. We found that DEPC-treated PMT is still able to compete with wt-PMT most likely at the receptor binding site. Therefore, we suggest that DEPC modifies catalytically important histidine residues in the C-terminal part of PMT.

We next studied the effect of mutation of all histidine residues, within the C-terminal 704-amino acids of PMT. We mutated the histidines to leucine or alanine, and we tested each mutant toxin for correctly folding by tryptic digestion. The tryptic digestion profiles of the mutants were similar to wt-PMT.

The results from PLC activation, neurite retraction, and ERK 1/2 activation studies of the toxin mutants showed that two histidine residues (H1205 and H1223) are essential for its biological activity in all three assays. These results suggest that it is unlikely that PMT harbors two different active sites such as that found for ExoS from *Pseudomonas aeruginosa* (*25*, *26*). We observed a smaller PLC activation and a delayed neurite retraction induced by PMT.H1228L. This diminished activity of PMT.H1228L was also detected in the ERK 1/2 activation assay. In addition, we found that PMT.H1205L also inhibited wt-PMT in competition experiments (Figure 6).

There are now three essential amino acid residues identified for PMT activity, the cysteine at position 1165 and two histidines at positions 1205 and 1223. Mutation of a third histidine (H1228) showed reduced PMT activity by a factor of 3. The results suggest the location of a potential metal ion-binding site at the C-terminus, consisting of H1205, H1223, and perhaps C1165. Although we could not find any conserved metal ion-binding motif, and the chelators TPEN, 2,2′-dipyridyl and L-penicillamine were without effect (not shown), the inhibition of PMT581C by 1,10-phenanthroline but not by the nonchelating 1,7-phenanthroline suggests this may be possible. In previous studies, a similar speculation about a potential metal ion-binding domain was reported. Petersen (*27*), who investigated the nucleotide sequence of the PMT gene, proposed that the cluster of cysteine and histidine residues between positions 1158 and 1229 could provide a site for metal ion binding (*22*). Additionally, it has been reported that the production and the lethality of PMT is affected by different cations (*28*). In fact, precise knowledge of the type of metal bound to the toxin would help to elucidate the mechanism by which PMT alters eukaryotic cell functions.

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