Action of *Pasteurella multocida* Toxin Depends on the Helical Domain of Goq*

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**Pasteurella multocida** produces a 146-kDa protein toxin (PMT), which activates multiple cellular signal transduction pathways, resulting in the activation of phospholipase Cβ, RhoA, Jun kinase, and intercellular signal-regulated kinase. Using Goq/Go11-deficient cells, it was shown that the PMT-induced pleiotropic effects are mediated by Goq, but not by the highly related Go11 protein (Zywietz, A., Gohla, A., Schmelz, M., Schultz, G., and Offermanns, S. (2001) *J. Biol. Chem.* 276, 3840–3845). Here we studied the molecular basis of the unique specificity of PMT to distinguish between Goq and/or Go11. Infection of Goq, Go11-deficient cells with retrovirus-encoding Goq caused reconstitution of PMT-induced activation of phospholipase Cβ, whereas Go11-encoding virus did not reconstitute PMT activity. Chimeras between Goq and Go11 revealed that a peptide region of Goq, covering amino acid residues 105–113, is essential for the action of PMT to activate phospholipase Cβ. Exchange of glutamine 105 or asparagine 109 of Goq, which are located in the all-helical domain of the Go subunit, with the equally positioned histidines of Goq, renders Go11 capable of transmission PMT-induced phospholipase Cβ activation. The data indicate that the all-helical domain of Goq is essential for the action of PMT and suggest an essential functional role of this domain in signal transduction via Gq proteins.

The *Pasteurella multocida* is a facultative pathogen, which cause bite wound infections, pneumonia, endocarditis, and septicemia in men. In pigs, the pathogen induces atrophic rhinitis, which is characterized by a loss of nasal turbinate bone (1, 2). The 146-kDa protein toxin *P. multocida* toxin (PMT)*1* is the major virulence factor of the pathogen, the causative agent of atrophic rhinitis, and is responsible for the osteolytic activity of bacteria (1, 3–5). PMT consists of 1285 amino acid residues. It is generally accepted that the toxin is structured according to a typical AB toxin. Initial studies revealed that the N terminus of PMT is involved in the binding and in translocation of the toxin into target cells (6), whereas the biologically active domain is located in the C-terminal part of the protein (6, 7). This concept is in line with a significant sequence similarity of PMT at its N terminus with the N-terminal part of the cytotoxic necrotizing factor of *Edercherichia coli* that is also involved in binding and translocation. According to the hypothesis that the C terminus of PMT carries the biological activity, an essential cysteine residue (Cys1165) was identified at the C terminus (8). The change of Cys1165 to serine blocked toxin activity but not cell binding. In addition, histidine residues (His1205 and His1223) in this part of the toxin were recognized to be essential for the activity of PMT (9).

PMT activates numerous cellular signal transduction pathways. It is a strong mitogen and stimulates DNA synthesis and proliferation in several cell lines (10–14). The mitogenic actions of PMT appear to depend on the stimulation of the extracellular signal-regulated kinase (ERK) (15). PMT stimulates phospholipase Cβ1 (PLCβ1) in a Gq-dependent manner (16), resulting in calcium mobilization, accumulation of diacylglycerol, and activation of protein kinase C (11). In addition, PMT activates the small GTPase RhoA, thereby inducing formation of stress fibers, focal adhesions, and tyrosine phosphorylation of focal adhesion kinase and paxillin (12, 17). The activation of Rho, extracellular signal-regulated kinase, and Jun kinase appears to be independent of Goq, indicating that PMT activates signaling pathways in a Goq-dependent and -independent manner (18). So far, however, the precise mode of molecular action of PMT is not known.

Recently, it was shown by gene deletion of the α-subunits of Gq and Go11 that PMT acts on PLCβ via Goq, but not via Go11 (18). This is remarkable because Goq and Go11 are highly related and share 89% of their amino acid residues.

We studied the molecular basis for this unique difference between Goq and Go11. Using G protein chimeras, we identified the helical domain of Goq to be essential for mediating the activation of PLCβ by PMT. We obtained evidence that the region of helix αB of Goq is involved in the PMT effect. Moreover, exchange of residues Gln116 and Asn119 in Goq to that of Go11 renders Goq11 sensitive toward PMT effects. These findings show that not only the Ras-like GTPase domain of Goq, but also the helical domain, which is inserted into the GTP-binding domain before switch 1, are functionally important and essential for mediating the activating effects of PMT. These findings give important new information on the site of action of PMT and suggest that the all-helical domain is important for signal transduction processes from Goq to PLCβ.

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3. The abbreviations used are: PMT, *P. multocida* toxin; AGS, activating subunit of the heterotrimeric G protein; Cys1165; Goq, α-subunits of the heterotrimeric G protein Goq; Go11, α-subunits of the heterotrimeric G protein Go11.

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**EXPERIMENTAL PROCEDURES**

**Materials**—1H-Labeled inositol was obtained from PerkinElmer Life Sciences (Dreieich, Germany). PCR primers were from MWG Biotech (Ebersberg, Germany) or from Qiagen Operon Europe (Cologne, Germany). The QuickChange kit was from Stratagene (Heidelberg, Germany). Inositol-free minimal essential medium was purchased from Cell Concepts (Unkirch, Germany).

**Plasmids and Retroviral Vector Construction**—The plasmids contain-

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The total amount of inositol phosphate was measured as described under “Experimental Procedures.” Data are given as fold induction over buffer control. A, expression of $G_{\alpha_q}$-encoding virus recovers PMT-induced inositol phosphate production. $G_{\alpha_q}$- or $G_{\alpha_{q11}}$-encoding retrovirus was produced, and $G_{\alpha_q}$-/ $G_{\alpha_{q11}}$-deficient cells were transduced with the resulting retrovirus as described under “Experimental Procedures.” $G_{\alpha_q}$- or $G_{\alpha_{q11}}$-deficient cells, transduced with $G_{\alpha_q}$ (filled rhombus) or $G_{\alpha_{q11}}$ (open rhombus)-encoding retrovirus were incubated for 6 h at the indicated concentrations of PMT. The total amount of inositol phosphate was measured as described under “Experimental Procedures.” Data are given as fold stimulation over buffer control. B, expression of $G_{\alpha_q}$ or $G_{\alpha_{q11}}$ in retroviral transduced $G_{\alpha_q}$-/ $G_{\alpha_{q11}}$-deficient cells was examined by Western blot. Shown is an immunoblot of RIPA extracts of $G_{\alpha_q}$-/ $G_{\alpha_{q11}}$-deficient cells. The immunoblot was performed as described under “Experimental Procedures.”

Analysis of Total Inositol Phosphate—Infected $G_{\alpha_q}$-/ $G_{\alpha_{q11}}$-deficient cells were grown in 24-well plates for 3 days and labeled with 2 $\mu$Ci/ml [2-3H]inositol in isositol- and serum-free medium (minimal essential medium) for 12 h. Subsequently, PMT and Lici (20 mM) were added, and the cells were incubated for the indicated times. For cell lysis and extraction of inositol phosphate, the medium was replaced with 750 $\mu$L of 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 phosphate was done by anion exchange chromatography using AG1-X8 resin (200–400 mesh; Bio-Rad, München, Germany) as described previously (7).

Expression and Purification of PMT Protein—Recombinant PMT protein was expressed as glutathione S-transferase fusion protein and purified according to the manufacturer’s instructions (Amersham Biosciences). In brief, glutathione S-transferase fusion protein was isolated by affinity chromatography with glutathione-Sepharose, followed by proteolytic cleavage using 3.25 units of thrombin/mg of recombinant glutathione S-transferase fusion protein. Thrombin was removed by incubation with benzonamide-Sepharose (Amersham Biosciences).

Western Blot Analysis—Wild-type, $G_{\alpha_q}$/ $G_{\alpha_{q11}}$-deficient, or retrovirus-infected cells were extracted at 4°C with RIPA buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl$_2$, 1 mM EDTA, 1% Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, COMPLETE$^\text{TM}$ protease inhibitors, Roche Applied Science) and analyzed by Western blotting after SDS-
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RESULTS

Fig. 1 shows the effects of PMT on wild-type and Goq/Go11-deficient cells. Whereas PMT causes a strong activation of inositol phosphate accumulation in wild-type cells, the toxin had no effect in Goq/Go11-deficient cells. The Western blot analysis revealed that wild-type cells expressed Goq and Go11, whereas in the Goq and Go11 gene-deficient cells, no expression of the a-subunits of Gq and G11 were detected. Next, we studied whether the PMT response could be reconstituted in Goq/Go11-deficient cells by introduction and expression of the cDNA of Gq or G11. As shown in Fig. 2, infection with Goq but not with Go11 caused reconstitution of the PMT effect. This confirms recent results showing that Goq but not Go11 is able to mediate PMT-induced activation of PLCβ.

To investigate the molecular mechanism responsible for this unique specificity of PMT, we decided to construct Goq/Go11 chimeras. Goq and Go11 are about 89% identical in their amino acid sequences. At first, we constructed a chimera consisting of the N-terminal part of residues 1–120 of Goq and 121–359 of Go11 (Goq-Go11121–359). Conversely, we constructed the chimera Goq-Go11359–121 (Fig. 3). These constructs were introduced into the retrovirus, and the Goq/Go11-deficient cells were infected with the retrovirus. As shown in Fig. 4, only the construct with the N-terminal part of Gq, e.g. Goq-Go11121–359, caused activation of PLCβ after PMT addition to infected cells.

Next, we further confined the region responsible for PMT sensitivity of Goq and constructed the chimera Goq-Go1167–120 (Fig. 3). As a control, we constructed the inverse chimera Goq-Go1167–120. Western blot analysis showed that these proteins were expressed in infected cells (Fig. 5). When cells, which expressed these chimeric G proteins, were treated with PMT, an increase in inositol phosphate accumulation was observed with Goq-Go1167–120 but not with the other constructs. This finding indicated that the structural requirements for mediation of PMT activity are located within a peptide of Gq that covers residues 67–120. This region harbors the helix aA and aB of the all-helical domain, and only a few residues are different between Goq and Go11 (Fig. 6A). To identify the region essential for PMT-mediated PLCβ activation, we exchanged helix aB of Goq and Go11 by sequential site-directed mutagenesis. Thereby, we generated chimeric Goq protein, harboring helix aB from Go11 (Goq-Go11105–113), and Go11 protein, harboring helix aB from Goq (Go11-Goq105–113) (Fig. 3). Both constructs were tested for the ability to mediate PMT-induced activation of PLCβ. As Fig. 6B shows PMT-stimulated PLCβ only via Go11-Goq105–113

Next, we exchanged glutamine at position 105 and aspara-
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Fig. 6. Introduction of helix aB of Goq into Go\(_{11}\) is sufficient to mediate PMT-induced activation of the resulting Goq/Go\(_{11}\) chimera. A, an alignment of Goq and Go\(_{11}\) harboring the N-terminal aA and aB helices of the helical domain is shown. The regions with predicted helical structures are indicated, and amino acids, which are different in Goq and Go\(_{11}\), are highlighted. In the region of helix aB four amino acids differ. These amino acids were sequentially exchanged in Goq and Go\(_{11}\) by site-directed mutagenesis as described under “Experimental Procedures.” B, retrovirus encoding for Go\(_{11}\)–Go\(_{q}\) (filled triangles) and Go\(_q\)–Go\(_{11}\) (open triangles) were generated and used to transduce Goq/Go\(_{11}\)-deficient cells. The ability to induce inositol phosphate formation by treatment with PMT was determined as before. C, expression of Go\(_{11}\)–Go\(_{q}\) or Go\(_q\)–Go\(_{11}\) in retroviral transduced Goq/Go\(_{11}\)-deficient cells was examined by Western blot. Shown is an immunoblot of RIPA extracts of Goq/Go\(_{11}\)-deficient cells. The immunoblot was performed as described under “Experimental Procedures.”

Fig. 7. Exchange of single amino acid residues in helix aB of Go\(_{11}\) to corresponding amino acid residues of Goq enables the resulting Goq/Go\(_{11}\) construct to mediate PMT signaling. A, retroviral transfer vector with inserts for Goq or Go\(_{11}\) was mutated by site-directed mutagenesis to generate the following constructs: Go\(_{11}\)Q105H (rhombus), Go\(_{11}\)N109H (squares) and GoqH105Q/H109N (triangles). Goq/Go\(_{11}\)-deficient cells, transduced with retrovirus encoding for described constructs, were used to test the ability to activate PLC\(\beta\) by PMT treatment. B, expression of Go\(_{11}\)Q105H, GoqN109H, and GoqH105Q/H109N in retroviral transduced Goq/Go\(_{11}\)-deficient cells was examined by Western blot. Shown is an immunoblot of RIPA extracts of Goq/Go\(_{11}\)-deficient cells. The immunoblot was performed as described under “Experimental Procedures.”
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tively (29, 30). Low molecular mass GTPases, especially of the Rho/Ras family, are modified by ADP-ribosylation, glucosylation, (e.g. Clostridium difficile toxins), and by deamidation (e.g. cytotoxic necrotizing factors from E. coli) (28, 31). Moreover, several bacterial effectors activate or inactivate small GTPases by mimicking the activity of eukaryotic guanine nucleotide exchange factors (e.g. SopE from Salmonella enterica) and GTPase-activating proteins (e.g. SpIP from S. enterica) (28, 32, 33). So far no evidence is available that PMT belongs to one of these toxin families. Recently, it was shown that PMT induces tyrosine phosphorylation of Goq (34). Tyrosine phosphorylation of Goq, has been claimed to regulate the activity of the G protein (35, 36). However, PMT-induced tyrosine phosphorylation of Goq, which occurs at the very C terminus, is also observed with the cysteine mutant that is defective in activation of PLCB (34).

Here, we report that exchange of only one amino acid residue in Goq11 (e.g. Gln106 or Asn109) allows PMT-induced activation of PLCB. Surprisingly, the reverse is not true; exchange of the equivalent residues in Goq9 (e.g. His105 or His109) did not block its ability to mediate PMT-induced activation of PLCB. One explanation of this finding is that the interaction of PMT (or that of a putative PMT-effector) with Goq9 and the subsequent activation of the G protein is supported by several amino acid residues, some of which are located in the a/b helix. Exchange of one of these residues did not prevent the PMT effect. However, only one of these crucial residues allows mediation of PMT activation of PLCB in Goq11.

Taken together, our data indicate that the a-helical region, especially the a/b helix, is essential for the functional difference of Goq9 and Goq11 to serve as a target of PMT. These data support the view that the helical domain of Goq9 has important functions in G protein activation and signaling.

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