Substrate specificity of *Pasteurella multocida* toxin for α subunits of heterotrimeric G proteins

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ABSTRACT **_Pasteurella multocida_ is the causative agent of a number of epizootic and zoonotic diseases. Its major virulence factor associated with atrophic rhinitis in animals and dermonecrosis in bite wounds is _P. multocida_ toxin (PMT). PMT stimulates signal transduction pathways downstream of heterotrimeric G proteins, leading to effects such as mitogenicity, blockade of apoptosis, or inhibition of osteoblast differentiation. On the basis of $G\alpha_{i2}$, it was demonstrated that the toxin deamidates an essential glutamine residue of the $G\alpha_{i2}$ subunit, leading to constitutive activation of the G protein. Here, we studied the specificity of PMT for its G-protein targets by mass spectrometric analyses and by utilizing a monoclonal antibody, which recognizes specifically G proteins deamidated by PMT. The studies revealed deamidation of 3 of 4 families of heterotrimeric G proteins ($G\alpha_{q/11}$, $G\alpha_{i1,2,3}$, and $G\alpha_{12/13}$ of mouse or human origin) by PMT but not by a catalytic inactive toxin mutant. With the use of G-protein fragments and chimeras of responsive or unresponsive G proteins, the structural basis for the discrimination of heterotrimeric G proteins was studied. Our results elucidate substrate specificity of PMT on the molecular level and provide evidence for the underlying structural reasons of substrate discrimination.—Orth, J. H. C., Fester, I., Siegert, P., Weise, M., Lanner, U., Kamitani, S., Tachibana, T, Wilson, B. A., Schlosser, A., Horiguchi, Y., Aktories, K. Substrate specificity of _Pasteurella multocida_ toxin for α subunits of heterotrimeric G proteins. _FASEB J._ 27, 832–842 (2013). www.fasebj.org**

Key Words: PMT · GTPase domain · helical domain · deamidation · oncogene

THE 146-kDa TOXIN FROM *Pasteurella multocida* (PMT) is a major virulence factor responsible for a number of the severe symptoms associated with various zoonotic and epizootic diseases in wild and domestic animals, including pasteurellosis and bite-wound dermonecrosis. In swine and rabbits, PMT exposure leads to atrophic rhinitis, which is characterized by destruction of the nasal turbinates (1–3). Intoxication of mammalian cells by PMT leads to increased total inositol phosphate levels due to activation of phospholipase Cβ (PLCβ; ref. 4). PMT exhibits strong mitogenic (5, 6) and antiapoptotic effects in various cell lines (7) and alters gene expression by activation of calcium (8), mitogen-activated protein (MAP) kinase (9, 10), and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) (11, 12) signaling pathways. A number of these pathways are involved in tumorigenesis, in particular those leading to sustained proliferative signaling or impaired apoptosis (13). These special features of PMT action led to the hypothesis of a link between bacterial toxins and cancer (14, 15). The cellular effects of PMT are induced by the activation of heterotrimeric G proteins of different families. Increased PLCβ activity is caused by PMT-induced activation of $G\alpha_q$ (4). Initial gene deletion studies and reconstitution experiments, using $G\alpha_{q/11}$-deficient mouse embryonic fibroblasts (MEFs), showed PMT-induced signaling *via* $G\alpha_q$, whereas the toxin did not activate PLCβ *via* the closely related $G\alpha_{11}$ (10, 16). In addition to activation of $G\alpha_q$, PMT can inhibit adenylyl cyclase activity through activation of $G\alpha_i$ (17) and can induce RhoA-mediated actin stress fiber formation through activation of $G\alpha_{13}$ of the

Abbreviations: GEF, guanine nucleotide exchange factor; HD, helical domain; MEF, mouse embryonic fibroblast; MS, mass spectrometry; PLCβ, phospholipase Cβ; PMT, *Pasteurella multocida* toxin; Q-TOF, quadrupole time-of-flight; WT, wild type

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$G_{12/13}$ family (18); however, it was not determined whether PMT could discriminate between the G_{12} and G_{13} proteins.

PMT is an AB toxin, consisting of a receptor binding and translocation domain at the N terminus and a biological active domain at the C terminus (19, 20). The crystal structure of a C-terminal fragment of PMT (aa 569–1285) revealed 3 domains (C1–C3) (21). Whereas the function of domain C2 is still enigmatic, domain C1 is involved in membrane targeting (22), and domain C3 is responsible for the biological activity (21, 23). The latter domain contains an active site catalytic triad, consisting of the essential amino acids Cys-1165 (19, 24), His-1205 (25, 26), and Asp-1220. Recently, we identified the molecular mechanism of PMT as the deamidation of a specific Gln residue, which is essential for catalyzing GTP hydrolysis in the α subunits of targeted G proteins (27). The resulting deamidation by PMT constitutively activates the α subunit of heterotrimeric G proteins.

The family of heterotrimeric G proteins consists of 4 major families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (28). So far, the substrate specificity of PMT was deduced indirectly from the effects of PMT on G-protein-dependent signal transduction, *e.g.*, PMT-induced activation of PLCβ1 (4), activation of RhoA (18), or inhibition of adenylyl cyclase (17). To unambiguously clarify the substrate specificity of PMT, we have now analyzed the PMT-induced deamidation of Gα subunits directly by a combination of mass spectrometry (MS) and immunoblot analysis using a monoclonal antibody, which selectively recognizes the deamidated Gln in the switch II region of Gα proteins (29). To further refine substrate specificity of PMT, we studied the effects of PMT on Gα-protein chimeras and on fragments of $G\alpha_{i2}$, consisting of the Ras-like GTPase domain only.

MATERIALS AND METHODS

Materials

PCR primers were from Apara (Denzlingen, Germany). All other reagents were of analytical grade and purchased from commercial sources.

Cell culture, transfection, virus production, and transduction

HEK-293 cells were transfected using PEI as described previously (30). MEFs derived from wild-type (WT) or $G\alpha_{q/11}$-gene-deficient mice were cultured as described previously (31, 32). The retroviral vector was produced as described previously (18). In brief, HEK-293T cells were cotransfected with pMD-G, pMD-g/p, and the retroviral transfer vector, using the calcium phosphate method. The supernatant was collected after 3 d and centrifuged to spin down cellular debris. The virus-containing medium was filtered. Cells were infected in the presence of polybrene. Protein expression was monitored by immunoblot analysis. Caco-2 cells were cultured in DMEM supplemented with 10% FCS, 1% nonessential amino acids, and 1% sodium pyruvate.

Plasmids and retroviral vector construction

cDNA of mouse $G\alpha_{11}$ was from Dr. B. Nürnberg (Universität Tübingen, Tübingen, Germany), and cDNA of bovine $G\alpha_s$ was from Dr. C. Kleuss (Freie Universität Berlin, Berlin, Germany). The plasmids pMD-G and pMD-g/p (33) were kindly provided by Dr. R. Mulligan (Harvard Medical School, Boston, MA, USA). The plasmid pLNCX2 was purchased from Clontech (Heidelberg, Germany). The pcDNA3.1 mammalian expression vectors, encoding human $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_s$, $G\alpha_{11}$, $G\alpha_{12}$, and $G\alpha_{13}$, were obtained from Missouri S&T cDNA Resource Center (http://www.cDNA.org).

The plasmid encoding the chimera $G\alpha_q$-$G\alpha_s$ ($G\alpha_q^{1–216}$-$G\alpha_s^{221–380}$; human $G\alpha_q$; and bovine $G\alpha_s$) was generated by splicing using overlap extension and cloned into pcDNA3.1 without the His tag. Only one of the two complementary overlapping primers used for the chimera is listed: $G\alpha_q^{1–216}$-$G\alpha_s^{221–380}$, 5′-TCAGAGAGAAGAAAATGGATCCAATGCTTCAATGAT-3′.

$G\alpha_{i1}$ and $G\alpha_{i3}$ were cloned into the bacterial expression vector pET41a by standard cloning techniques. The mutations in the previously described $G\alpha_{i2}$-pET41a vector (27) were introduced by site-directed mutagenesis. Only one of the complementary primers used is listed: $G\alpha_{i2}^{S207D}$, 5′-GGTGGT-CAGCGGGATGAGCGGAAGAAGTGG-3′; $G\alpha_{i2}^{E208Q}$, 5′-GGT-CAGCGGTCTCAACGGAAGAGGTGGATC-3′.

Fragments of $G\alpha_{i2}$, deletion of the helical domain (HD; $G\alpha_{i2}^{\Delta HD}$) and the switch II region of $G\alpha_{i2}$ ($G\alpha_{i2}^{swII}$), were cloned into the pET41a vector by standard PCR techniques. $G\alpha_{i2}^{\Delta HD}$ consists of the GTPase domain, whereas the HD (residues 61–175) was replaced by a spacer coding for Ser-Ala-Gly-Ala. $G\alpha_{i2}^{swII}$ comprises the region directly around the switch II region (residues 184–218).

An internal flag tag was introduced at position 139 of $G\alpha_{13}$ by replacing Thr-139 or at position 121 of $G\alpha_{i1}$ by replacing Thr-121 with the sequence SGGGGYPDYKDDDDKGGGGS, using standard PCR cloning procedures.

MS analysis

For in-gel digestion, the excised gel bands were destained with 30% acetonitrile, shrunk with 100% acetonitrile, and dried in a vacuum concentrator (Concentrator 5301; Eppendorf, Hamburg, Germany). Digests with trypsin were performed overnight at 37°C in 0.05 M NH_4HCO_3 (pH 8). About 0.1 μg of protease was used for each gel band. Peptides were extracted from the gel slices with 5% formic acid. All LC-MS/MS analyses were performed with the 1200 Agilent Chip-HPLC system (Agilent Technologies, Böblingen, Germany), either coupled to a quadrupole time-of-flight (Q-TOF; Agilent 6520) or an ion trap (Agilent 6340) mass spectrometer. Peptides were separated on the HPLC-Chip with an analytical column of 75 μm inner diamter and 150 mm length and a 40 nl trap column, both packed with Zorbax 300SB C-18 (5 μm particle size; Agilent). Peptides were eluted with a linear acetonitrile gradient with 1%/min at a flow rate of 300 nl/min (starting with 3% acetonitrile). The Q-TOF spectrometer was operated in the 2 GHz extended dynamic range mode. MS/MS analyses were performed using data-dependent acquisition mode. After a MS scan (2 spectra/s), a maximum of 3 peptides were selected for MS/MS (2 spectra/s). Singly charged precursor ions were excluded from selection. Internal calibration was applied using one reference mass. LC-MS/MS analyses on the ion trap were performed using data-dependent acquisition mode. After a MS scan (standard enhanced mode), a maximum of 3 peptides were selected for MS/MS (collision-induced dissociation, standard enhanced mode). The automated gain control was set to 350,000. The maximum accumulation time was set to 300 ms.

Protein expression

Expression of WT or catalytically inactive mutants (C1165S) of PMT or PMT fragment (PMT-C) or coexpression with $G\alpha_{i1}$, $G\alpha_{i3}$, or $G\alpha_{i2}$ mutants and deletions was performed as described previously (19, 27).

Immunoprecipitation of Gα proteins

Cells were washed twice with cold PBS and lysed in 50 mM Na-HEPES (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and complete protease inhibitor cocktail (Roche, Mannheim, Germany) at 4°C for 1 h. After centrifugation at 3500 *g* for 10 min at 4°C, the supernatant was used for immunoprecipitation. To remove proteins that bind nonspecifically to protein G-Sepharose, lysates were incubated with protein A/G-Sepharose (sc-2003; Santa Cruz Biotechnology, Heidelberg, Germany) for 30 min at 4°C. After centrifugation at 1000 *g* for 1 min, the supernatant was used for immunoprecipitation by incubation overnight at 4°C with the indicated antibody and protein G-Sepharose 4B (096K1347; Sigma-Aldrich, Taufkirchen, Germany). Sepharose beads were collected and washed with lysis buffer. Bound proteins were released by treating the Sepharose beads with 15 μl 2× Laemmli-buffer for 30 min at 50°C and followed by 5 min at 85°C. Sepharose beads were separated from eluted proteins by centrifugation, and the proteins were further analyzed by SDS-PAGE and immunoblotting.

Immunoblot analysis

For immunoblotting, samples were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membrane. $G\alpha_q$, $G\alpha_{12}$, $G\alpha_s$, and $G\alpha_{q/11}$ antibodies were purchased from Santa Cruz Biotechnology. $G\alpha_i$ and $G\alpha_{pan}$ antibodies were obtained from Cell Signaling Technology (Frankfurt, Germany). Flag-tag (M2), and tubulin antibodies were purchased from Sigma-Aldrich. Deamidation-specific antibody anti-$G\alpha_q$ Q209E (3G3) was used as described previously (29). Binding of the appropriate horseradish peroxidase-coupled secondary antibody was detected by using enhanced chemiluminescent detection reagent and the LAS-3000 imaging system (Fujifilm, Düsseldorf, Germany).

RESULTS

PMT and $G\alpha_i$ family members

The $G\alpha_i$ family of heterotrimeric G proteins consists of $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$, *inter alia* (28). Since previous studies showed that PMT deamidates $G\alpha_{i2}$, leading to an inhibition of adenylyl cyclase (17, 27), we asked whether PMT also activates $G\alpha_{i1}$ and $G\alpha_{i3}$ by deamidation. To this end, an active fragment of PMT (PMT-C) was coexpressed in *Escherichia coli* with $G\alpha_{i1}$ or $G\alpha_{i3}$. The G-protein α subunits were tested for functionality by a nucleotide-binding assay and then subjected to MS analysis (**Fig. 1** and Supplemental Fig. S1). MS analysis identified a tryptic peptide of $G\alpha_{i1}$ corresponding to aa 198–205 (m/z 455.216^{2+}). This peptide included the Gln residue (Gln-204) crucial for GTP hydrolysis. A second peptide was also identified with a mass shift of 1 Da (m/z 455.708^{2+}). Tandem MS analysis identified a Glu residue at position 204, indicating that Gln-204 had been deamidated by PMT. Similar results were obtained for $G\alpha_{i3}$ (Fig. 1*B*). No relevant deamidation product was identified when $G\alpha_{i1}$ or $G\alpha_{i3}$ was expressed without PMT (Fig. 1*A*, *B*).

In addition, we utilized a recently described monoclonal antibody (29), which specifically recognizes the deamidated state of the Gln residue in position 204 of $G\alpha_i$. This anti-QE antibody was engineered to detect the switch II region of $G\alpha_q$ after PMT-induced deamidation of the crucial Gln residue. Due to the high sequence identity in the switch II region of Gα proteins, the anti-QE antibody also recognizes other deamidated forms of G-protein family members, such as $G\alpha_i$ or $G\alpha_s$. The specificity of the antibody was verified by using mutants of $G\alpha_i$ (*e.g.*, $G\alpha_{i1}^{Q204E}$), which mimic the toxin-induced deamidation (29). To study the substrate specificity of PMT toward $G\alpha_i$ family members, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ were ectopically expressed in HEK-293 cells. Gα-expressing cells were intoxicated with PMT, and cell lysates were analyzed by immunoblotting for deamidated G proteins. Overexpression of $G\alpha_{i1\text{-}3}$ largely increased the immunoblot signal by the QE-specific antibody, indicating a PMT-induced deamidation of the ectopically expressed G proteins (**Fig. 2*A***). Thus, the data indicate that PMT accepts all $G\alpha_i$ family members tested as substrates. Additionally, we performed a time course of PMT-induced deamidation. We treated Caco-2 cells for 1 h with the toxin and, subsequently, incubated the cells for up to 7 d. We detected deamidation of G proteins up to 6 d after initial PMT treatment (Fig. 2*B*).

PMT acts on the $G\alpha_{q/11}$ family

Activation of $G\alpha_q$-dependent signaling pathways is a consequence of PMT intoxication (4, 16). Therefore, we wanted to analyze the direct action of the toxin on the G proteins involved. For this purpose, $G\alpha_q$ was immunoprecipitated from PMT-treated cells by a specific antibody (**Fig. 3*C***), and then subjected to MS analysis of tryptic peptides, as described previously (27). As shown in Fig. 3*A*, Gln-209 of $G\alpha_q$ was deamidated by PMT intoxication. The deamidation of Gln-209 to Glu was verified by a 1-Da mass shift of the peptide and a retention time shift of ~1 min (m/z 431.2^{2+} 203-MVDVGG**Q**R-210; m/z 431.7^{2+} 203-MVDVG-G**E**R-210). In line with these findings are the corresponding MS/MS spectra (Supplemental Fig. S2).

$G\alpha_{11}$ also belongs to the $G\alpha_q$ family and shares 89% amino acid sequence identity with $G\alpha_q$. To avoid cross contamination of $G\alpha_{11}$ with $G\alpha_q$ during immunoprecipitation, we utilized MEF cells deficient for $G\alpha_q$ and $G\alpha_{11}$. In $G\alpha_{q/11}$-deficient MEFs, $G\alpha_{11}$ was expressed by retroviral transfection (Fig. 3*D*). Immunoprecipitation of $G\alpha_{11}$ from PMT-treated cells was performed (Fig. 3*E*). As was observed for $G\alpha_q$, MS analysis revealed deamidation of Gln-209 of $G\alpha_{11}$ (Fig. 3*B* and Supplemental Fig. S2), indicating that both $G\alpha_q$ and $G\alpha_{11}$ are targeted by PMT. These data are in contrast to previous results,

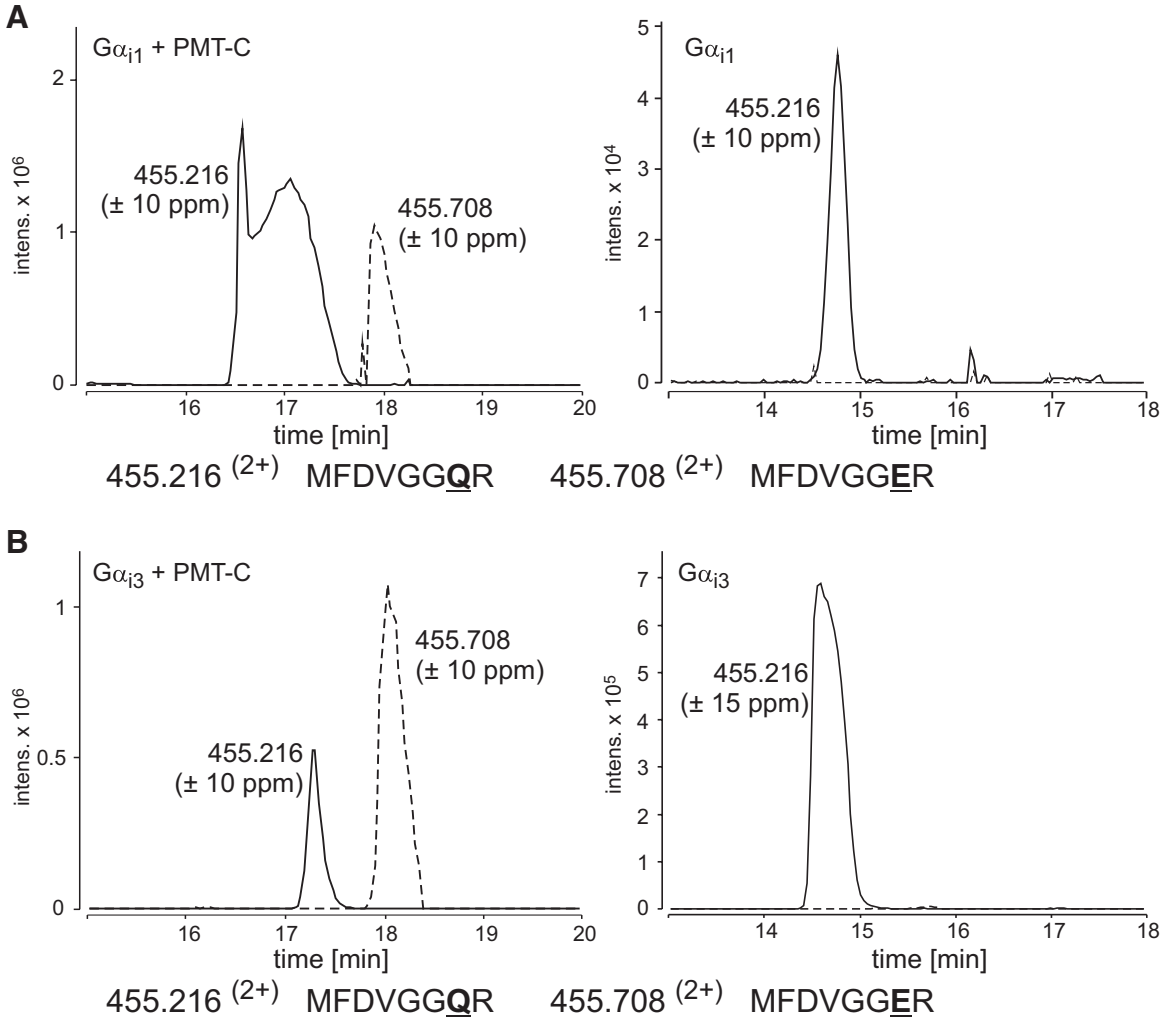

Figure 1. $G\alpha_i$ family members are substrates of PMT. $G\alpha_i$ proteins were coexpressed with an active PMT fragment and subjected to MS analysis. Left panels: combined extracted ion chromatograms (Q-TOF data) for *m/z* 455.216 (±10 ppm; solid line) and 455.708 (±10 ppm; dashed line), corresponding to the tryptic peptides MFDVGGQR and MFDVGGER (aa 198–205) of $G\alpha_{i1}$ (*A*, left panel) and $G\alpha_{i3}$ (*B*, left panel). The presence of the tryptic peptide MFDVGGER indicates deamidation of $G\alpha_{i1}$ and $G\alpha_{i3}$ at the essential Gln-204 by PMT. Right panels: no relevant deamidation product was detectable when $G\alpha_{i1}$ (*A*) and $G\alpha_{i3}$ (*B*) were expressed without PMT.

suggesting that only $G\alpha_q$- but not $G\alpha_{11}$-mediated signaling is affected by PMT (10, 16).

$G\alpha_{12/13}$ are substrates of PMT

RhoA is activated by $G\alpha_{12/13}$- and $G\alpha_{q/11}$-dependent signaling pathways (32, 34). The Gα subunits cause activation of specific RhoA guanine nucleotide exchange factors (GEFs), such as p115RhoGEF or p63RhoGEF, which are specific for $G\alpha_{12/13}$ or $G\alpha_{q/11}$, respectively (35, 36). Previously, we reported the involvement of $G\alpha_{12/13}$ in PMT-induced activation of RhoA (18). However, these studies did not permit distinction between $G\alpha_{12}$ and $G\alpha_{13}$ as substrates of PMT. To determine whether $G\alpha_{12}$ and/or $G\alpha_{13}$ are targets of PMT, $G\alpha_{13}$ was ectopically expressed with an internal flag tag. $G\alpha_{13}$ was precipitated from PMT-treated or untreated cells by using an anti-flag antibody (**Fig. 4*B***). MS analysis of the immunoprecipitated flag-tagged $G\alpha_{13}$ subunits identified a 1-Da shift of 2 tryptic peptides, encompassing the amino acid residues 220 to 227 that harbor the essential Gln residue (control peptide precursor with *m/z* 431.216^{2+} (220-MVDVGGQR-227) and toxin-modified peptide precursor with *m/z* 431.708^{2+} (220-MVDVGGER-227). Tandem MS analysis confirmed deamidation of Gln-226 in the switch II region of $G\alpha_{13}$ to Glu (Fig. 4 and Supplemental Fig. S3).

In addition, we overexpressed $G\alpha_{12}$ (Fig. 4*C*) to clarify whether both members of the $G\alpha_{12/13}$ family are substrates of PMT. MS analysis of $G\alpha_{12}$ from PMT-intoxicated or untreated cells again revealed two tryptic peptides, encompassing amino acid residue 225 to 232, that harbor the crucial Gln-231 of the switch II region of $G\alpha_{12}$ (*m/z* 431.216^{2+} 225-MVDVGGQR-232; *m/z* 431.708^{2+} 225-MVDVGGER-232). Tandem MS analysis indicated deamidation of Gln-231 (Fig. 4 and Supplemental Fig. S3).

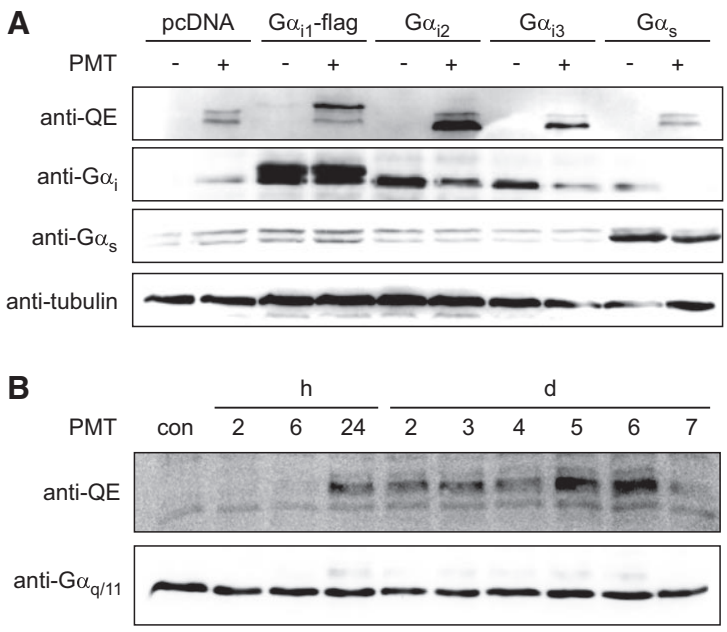

Figure 2. Determination of the substrate specificity of PMT using a monoclonal anti-QE antibody that detects toxin-deamidated Gα proteins. *A*) HEK-293 cells were transfected with pcDNA3-based plasmids expressing the indicated Gα subunits. After 24 h of incubation, the cells were treated with or without PMT (1 nM) for a further 24 h. Cells were lysed and subjected to SDS-PAGE, followed by immunoblotting with monoclonal rat anti-$G\alpha_q$ Q209E (3G3; anti-QE), polyclonal rabbit anti-$G\alpha_i$, polyclonal rabbit anti-$G\alpha_s$, and monoclonal mouse anti-tubulin antibody as described in Materials and Methods. Note that control HEK-293 cells (pcDNA) and recombinant $G\alpha_s$-expressing cells contain small amounts of endogenous G proteins, which are deamidated by PMT and labeled with anti-QE (double bands in top panel, lanes 2 and 10). The double bands labeled by anti-$G\alpha_s$ indicate the two isoforms of $G\alpha_s$. *B*) CaCo-2 cells were treated with PMT (1 nM) for 1 h. Toxin-containing medium was discarded, and cells were washed with medium and incubated for indicated times. Cells were lysed and subjected to SDS-PAGE, followed by immunoblotting with monoclonal rat anti-$G\alpha_q$ Q209E (3G3; anti-QE) and polyclonal rabbit anti-$G\alpha_{q/11}$ antibody.

G-protein mutants and fragments

The switch II region of α subunits of heterotrimeric G proteins is highly conserved throughout all G-protein family members, with only minor sequence differences (**Fig. 5*A***). We tested whether changes in this region might define the substrate specificity of PMT. $G\alpha_s$, which is not a target for PMT (see below), possesses an Asp two residues upstream of the crucial Gln, while the known PMT substrates have Ser at this position. We

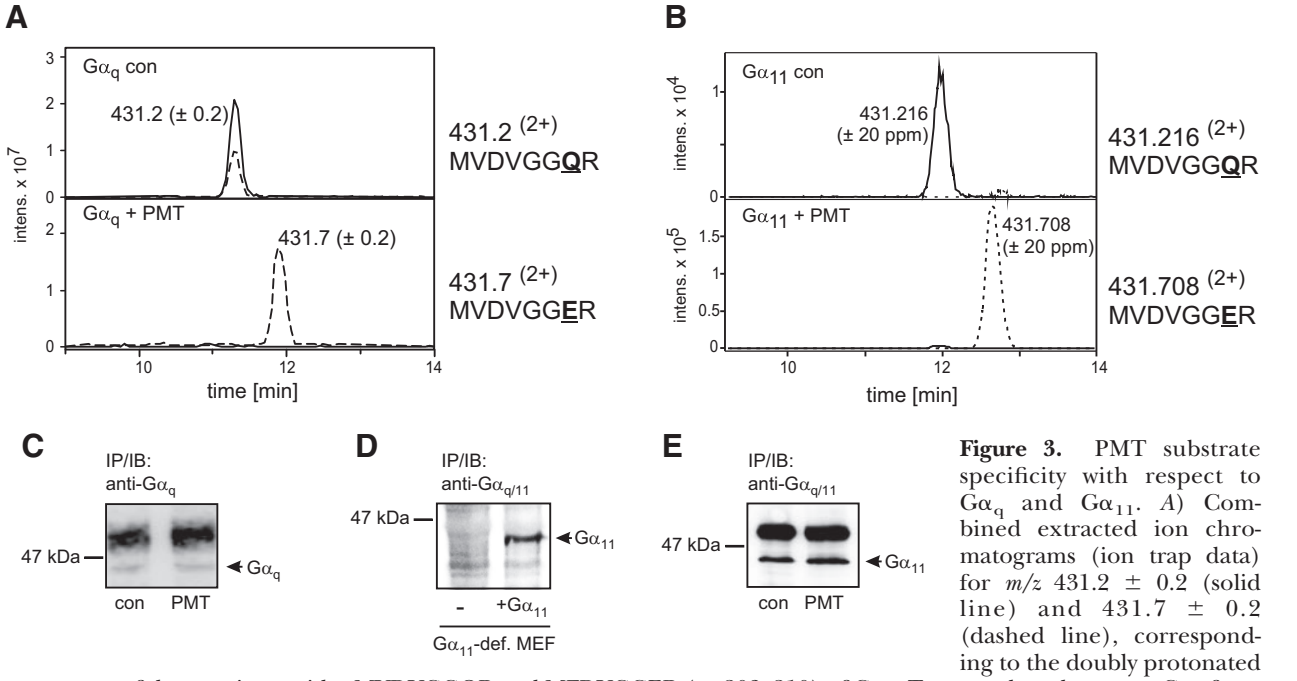

Figure 3. PMT substrate specificity with respect to $G\alpha_q$ and $G\alpha_{11}$. *A*) Combined extracted ion chromatograms (ion trap data) for *m/z* 431.2 ± 0.2 (solid line) and 431.7 ± 0.2 (dashed line), corresponding to the doubly protonated precursors of the tryptic peptides MVDVGGQR and MFDVGGER (aa 203–210) of $G\alpha_q$. Top panel: endogenous $G\alpha_q$ from untreated WT-MEFs. Bottom panel: endogenous $G\alpha_q$ from PMT-treated WT-MEFs. *B*) Combined extracted ion chromatograms (Q-TOF data) for *m/z* 431.216 ± 20 ppm (solid line) and 431.708 ± 20 ppm (dashed line), corresponding to the doubly protonated precursors of the tryptic peptides MVDVGGQR and MFDVGGER (aa 203–210) of $G\alpha_{11}$. $G\alpha_{11}$ was precipitated from $G\alpha_{q/11}$-deficient MEFs, which were transduced with retrovirus encoding for $G\alpha_{11}$.Top panel: $G\alpha_{11}$ from untreated cells. Bottom panel: $G\alpha_{11}$ from PMT-treated cells. Deamidation of Gln-209 of $G\alpha_q$ and $G\alpha_{11}$ is complete, and MVDVGGQR is no longer detectable after PMT treatment. *C*) Immunoblot of endogenous $G\alpha_q$ after immunoprecipitation with a specific $G\alpha_q$ antibody. Precipitations were performed from untreated MEF (con) or from PMT-treated MEF (1 nM, 18 h). *D*) Detection of $G\alpha_{11}$ in $G\alpha_{q/11}$-deficient MEFs (−) or in $G\alpha_{q/11}$-deficient MEFs transduced with $G\alpha_{11}$-encoding retrovirus (+$G\alpha_{11}$). *E*) Immunoblot of $G\alpha_{11}$ after immunoprecipitation with anti-$G\alpha_{q/11}$ antibody from $G\alpha_{q/11}$-deficient MEFs transduced with $G\alpha_{11}$-encoding retrovirus.

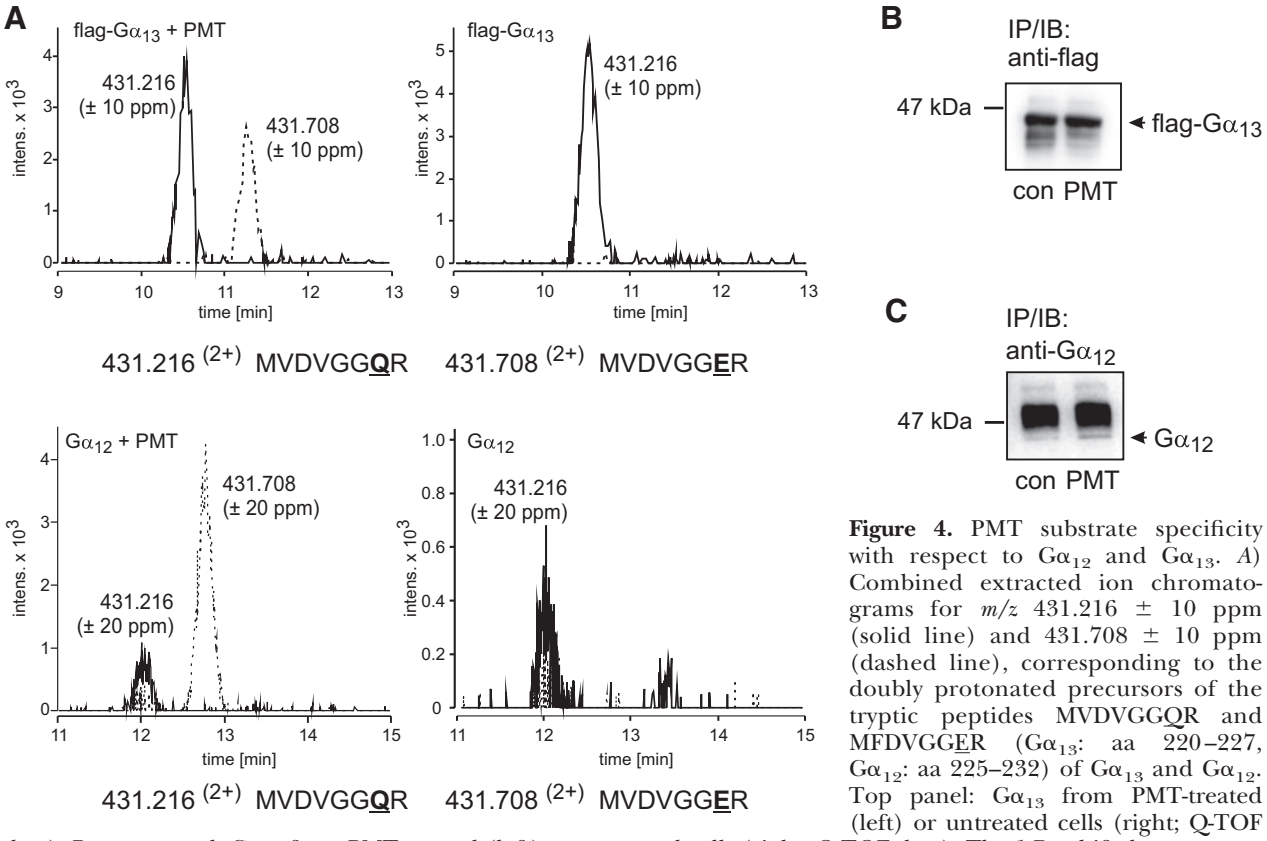

Figure 4. PMT substrate specificity with respect to $G\alpha_{12}$ and $G\alpha_{13}$. *A*) Combined extracted ion chromatograms for *m/z* 431.216 ± 10 ppm (solid line) and 431.708 ± 10 ppm (dashed line), corresponding to the doubly protonated precursors of the tryptic peptides MVDVGGQR and MFDVGGER ($G\alpha_{13}$: aa 220–227, $G\alpha_{12}$: aa 225–232) of $G\alpha_{13}$ and $G\alpha_{12}$. Top panel: $G\alpha_{13}$ from PMT-treated (left) or untreated cells (right; Q-TOF data). Bottom panel: $G\alpha_{12}$ from PMT-treated (left) or untreated cells (right; Q-TOF data). The 1-Da shift demonstrates the deamidation of Gln-226 ($G\alpha_{13}$) and Gln-231 ($G\alpha_{12}$) to Glu. The deamidated form of the tryptic peptide (MVDVGGER) is only detectable when $G\alpha_{13}$ or $G\alpha_{12}$ is precipitated from PMT-treated cells. In addition, unmodified peptide (MVDVGGQR) is present in precipitates from PMT-treated cells. *B*) $G\alpha_{13}$ cDNA was engineered to harbor an internal flag tag. The construct was ectopically expressed in HEK-293 cells. After treatment with or without PMT (1 nM, 18 h), cells were lysed, and flag-tagged protein was immunoprecipitated, isolated, and subjected to MS analysis. Shown is an immunoblot of $G\alpha_{13}$ after immunoprecipitation with anti-flag-tag antibody from PMT-treated or untreated (con) cells. *C*) $G\alpha_{12}$ was ectopically expressed in HEK-293 cells. After treatment with or without PMT (1 nM, 18 h), cells were lysed, and $G\alpha_{12}$ protein was immunoprecipitated, using a specific anti-$G\alpha_{12}$ antibody, isolated, and subjected to MS analysis. Immunoblot shows $G\alpha_{12}$ after immunoprecipitation with anti-$G\alpha_{12}$ antibody from PMT-treated or untreated (con) cells. The strong band above $G\alpha_{12}$ is part of the antibody used for immunoprecipitation.

changed the corresponding Ser-207 in $G\alpha_{i2}$ to Asp ($G\alpha_{\alpha i2}^{S207D}$). In addition, $G\alpha_{12}$, which is a target of PMT, has a unique Gln three residues upstream of the crucial Gln, whereas all other PMT substrates have Glu at the equivalent position. To detect any unspecific effect of mutations within the switch II region, we also replaced the corresponding Glu of $G\alpha_{i2}$ to Gln ($G\alpha_{\alpha i2}^{E208Q}$). The respective $G\alpha_{i2}$ mutants were coexpressed with the active C-terminal fragment of PMT (PMT-C^{wt}) in *E. coli* and were tested for functionality by a nucleotide-binding assay (Supplemental Fig. S4*A*). Tryptic peptides of the isolated G proteins were analyzed by HPLC and MS/MS. As shown in Fig. 5*B*, C (left panels), two peptides, 199-MFDVGGER-206 (*m/z* 455.708^{2+}) and 199-MFDVGGQR-206 (*m/z* 455.216^{2+}), were obtained by MS analysis from both mutants. The peptides differed by 1 Da, indicating the specific deamidation of Gln-205 of $G\alpha_{i2}$ by PMT (Supplemental Fig. S4). No relevant deamidation product was identified when $G\alpha_{\alpha i2}^{S207D}$ or $G\alpha_{\alpha i2}^{E208Q}$ was expressed without PMT (Fig. 5*B*, *C*; right panels). These results showed that both of the switch II mutant $G\alpha_{i2}$ proteins ($G\alpha_{\alpha i2}^{S207D}$ and $G\alpha_{\alpha i2}^{E208Q}$) were still substrates of PMT, indicating that the residues changed have no major effect on substrate recognition by PMT.

Because substrate specificity of PMT is dependent on the N terminus of Gα subunits, including their HDs, but not on the C terminus (37), which regulates the interaction with effectors, we next addressed whether the interaction of G proteins with specific effectors influences the action of PMT. To this end, we constructed a chimera of the PMT target $G\alpha_q$ with $G\alpha_s$, which is not a target of PMT (**Fig. 6**). The chimeric protein consisted of the N terminus of $G\alpha_q$, including the switch I and switch II regions of GTPase domain and the full HD, fused to the C terminus of the GTPase domain of $G\alpha_s$ ($G\alpha_q^{1-216}$-$G\alpha_s^{221-380}$; Fig. 6*A*). This chimera is still able to activate adenylyl cyclase *via* G_s-coupled receptors (38). PMT treatment of HEK-293 cells overexpressing the $G\alpha_q$-$G\alpha_s$ chimera but not $G\alpha_q$ nor $G\alpha_s$ increased cAMP production in a dose-dependent manner (Fig. 6*B*), indicating stimulation of adenylyl cyclase by PMT *via* activation of the $G\alpha_q$-$G\alpha_s$ chimera. We also observed that in pcDNA control

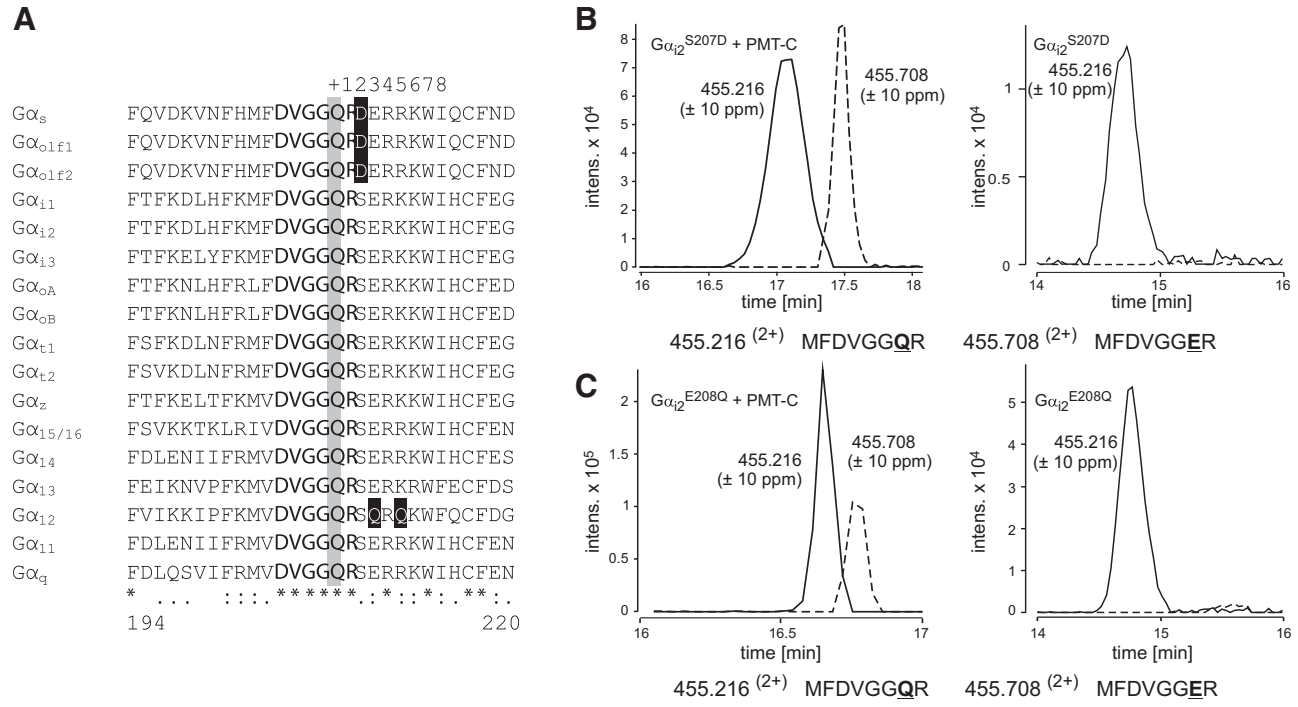

Figure 5. Switch II of G-protein α subunits is not involved in PMT substrate specificity. *A*) Alignment of amino acid sequences of the switch II region in the α subunits of mouse heterotrimeric GTPases. Nucleotide sequences are obtained from the U.S. National Center for Biotechnology Information (NCBI): $G\alpha_s$ (P63094), $G\alpha_{olf1}$ (NP_034437), $G\alpha_{olf2}$ (NP_796111), $G\alpha_{i1}$ (NP_034435), $G\alpha_{i2}$ (AAH65159), $G\alpha_{i3}$ (NP_034436), $G\alpha_{oA}$ (NP_034438), $G\alpha_{oB}$ (P18873.3), $G\alpha_{t1}$ (NP_032166), $G\alpha_{t2}$ (NP_032167), $G\alpha_z$ (NP_034441), $G\alpha_{15}$ (NP_034434), $G\alpha_{14}$ (NP_032163), $G\alpha_{13}$ (NP_034433), $G\alpha_{12}$ (NP_034432), $G\alpha_{11}$ (NP_034431), and $G\alpha_q$ (NP_032165). Numbers below the alignment correspond to the amino acid positions of $G\alpha_q$. The active site Gln (at position 209 in $G\alpha_q$) is indicated by a gray box, highly conserved flanking residues by boldface type, and flanking residues that result in notable charge differences by are indicated in reverse type on a black box. Asterisks denote identical amino acid residues; colons denote highly conserved residues; periods denote conserved residues. Numbers in the top row indicate positions downstream of the essential Gln. *B, C*) Combined extracted ion chromatograms (Q-TOF data) for *m/z* 455.216 ± 10 and 455.708 ± 10 ppm, corresponding to the tryptic peptides MFDVGGQR and MFDVGGER (aa 199–206) of $G\alpha_{i2}{}^{S207D}$ (*B*) and $G\alpha_{i2}{}^{E208Q}$ (*C*). $G\alpha_{i2}$ mutants were coexpressed with an active PMT fragment and subjected to MS analysis. The presence of the tryptic peptide MFDVGGER indicates deamidation of $G\alpha_{i2}{}^{S207D}$ and $G\alpha_{i2}{}^{E208Q}$ at Gln-205 by PMT. No relevant deamidation product was detectable when $G\alpha_{i2}{}^{S207D}$ and $G\alpha_{i2}{}^{E208Q}$ were expressed without PMT (right panels).

transfectants, basal adenylyl cyclase activity was inhibited by PMT. This is in line with our previous findings, showing that PMT activates $G\alpha_i$ to inhibit adenylyl cyclase activity (17, 27). The data obtained with the $G\alpha_q$-$G\alpha_s$ chimera suggest that PMT activation overrode the inhibition of adenylyl cyclase caused by toxin-induced activation of $G\alpha_i$. To verify that the observed activation of adenylyl cyclase was indeed due to PMT-induced deamidation of the $G\alpha_q$-$G\alpha_s$ chimera, the chimera was immunoprecipitated from toxin-treated HEK-293 cells (Fig. 6*C*) and analyzed by LC-MS/MS, which revealed that PMT catalyzed the deamidation of Gln-209 of the $G\alpha_q$-$G\alpha_s$ chimera (Fig. 6*D* and Supplemental Fig. S5). Two peptides were identified, corresponding to the unaffected chimera (*m/z* 431.216^{2+}) and the toxin-deamidated form (*m/z* 431.708^{2+}). Altogether, these results confirmed the involvement of the N terminus of Gα subunits in substrate recognition by PMT. We further tested whether $G\alpha_s$ could serve as a target of PMT. To this end, $G\alpha_s$ was immunoprecipitated from PMT-treated MEFs and subjected to LC-MS/MS analysis, but no deamidation product was detectable (Fig. 6*E, F* and Supplemental Fig. S5). In line with this finding, we did not detect deamidation of $G\alpha_s$ overexpressed in HEK-293 cells by using the QE-antibody (Fig. 2*A*). Both results confirm previous findings that $G\alpha_s$ is not a substrate of PMT.

To further define the minimal region of the G proteins that can serve as a substrate for PMT, fragments of $G\alpha_{i2}$ were constructed (**Fig. 7**). In $G\alpha_{i2}{}^{\Delta HD}$, the HD insert was deleted and consists therefore only of the GTPase domain of $G\alpha_{i2}$. $G\alpha_{i2}{}^{swII}$ comprises the amino acid residues of the switch II region. $G\alpha_{i2}$ and both constructs were coexpressed in *E. coli* with the active C-terminal fragment of PMT (PMT-C^{wt}) or the catalytically inactive PMT fragment (PMT-C^{C1165S}). Purified $G\alpha_{i2}$ mutant proteins were subjected to immunoblot analysis to detect deamidation of the essential Gln by PMT (Fig. 7*B*). $G\alpha_{i2}$, $G\alpha_{i2}{}^{\Delta HD}$, and $G\alpha_{i2}{}^{swII}$ were detected by the anti-$G\alpha_{pan}$ antibody. $G\alpha_{i2}$ and $G\alpha_{i2}{}^{\Delta HD}$ coexpressed with active PMT-C^{wt}, but not with PMT-C^{C1165S}, were recognized by the anti-QE antibody. $G\alpha_{i2}{}^{swII}$ was not detected by the QE-specific antibody under either condition. $G\alpha_{i2}{}^{\Delta HD}$ or $G\alpha_{i2}{}^{swII}$ coexpressed with WT or inactive PMT were also examined by mass spectral analysis (Fig. 7*C* and Supplemental Fig.

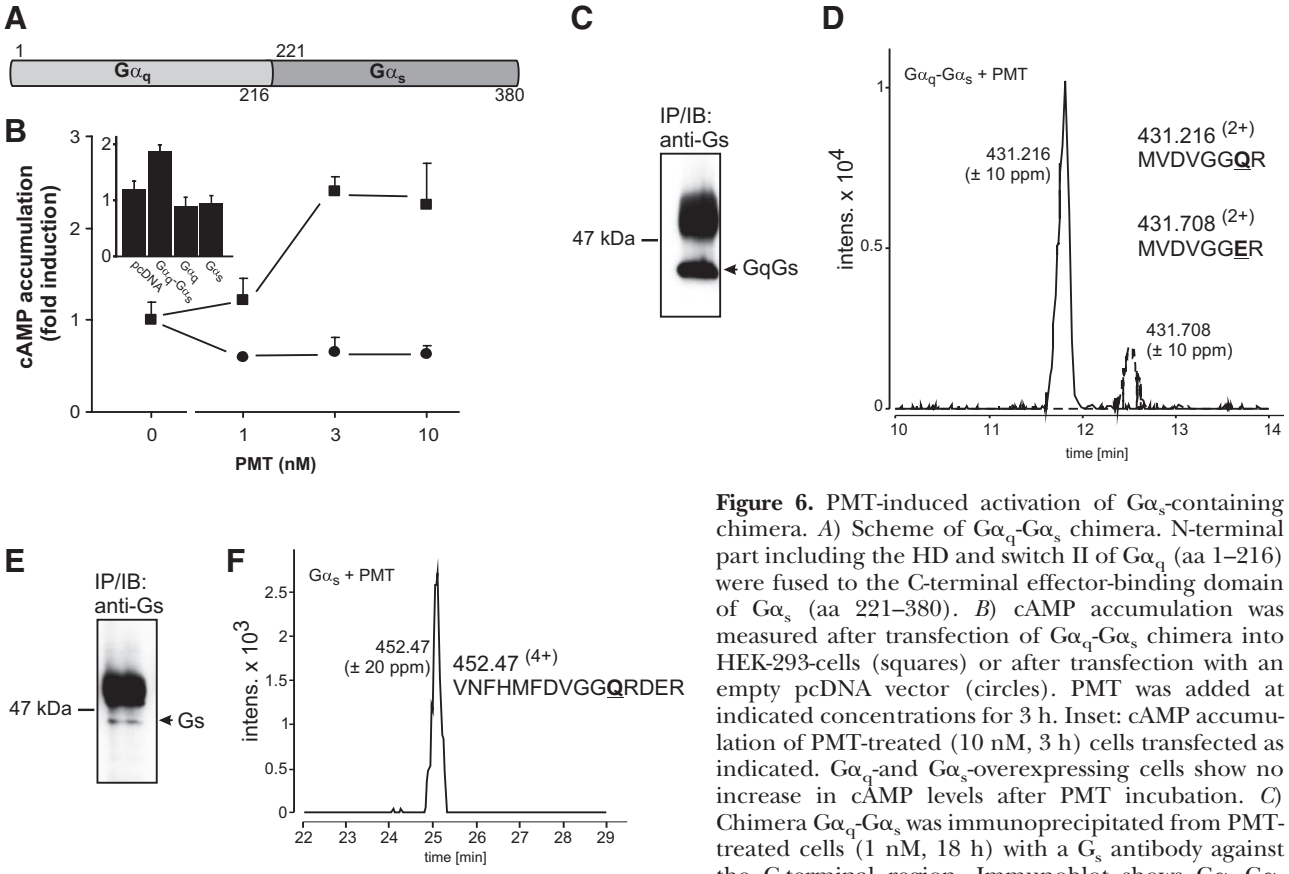

Figure 6. PMT-induced activation of $G\alpha_s$-containing chimera. *A*) Scheme of $G\alpha_q$-$G\alpha_s$ chimera. N-terminal part including the HD and switch II of $G\alpha_q$ (aa 1–216) were fused to the C-terminal effector-binding domain of $G\alpha_s$ (aa 221–380). *B*) cAMP accumulation was measured after transfection of $G\alpha_q$-$G\alpha_s$ chimera into HEK-293-cells (squares) or after transfection with an empty pcDNA vector (circles). PMT was added at indicated concentrations for 3 h. Inset: cAMP accumulation of PMT-treated (10 nM, 3 h) cells transfected as indicated. $G\alpha_q$-and $G\alpha_s$-overexpressing cells show no increase in cAMP levels after PMT incubation. *C*) Chimera $G\alpha_q$-$G\alpha_s$ was immunoprecipitated from PMT-treated cells (1 nM, 18 h) with a G_s antibody against the C-terminal region. Immunoblot shows $G\alpha_q$-$G\alpha_s$ chimera after immunoprecipitation with anti-Gs antibody. *D*) Combined extracted ion chromatograms (Q-TOF data) for *m/z* 431.216 ± 10 and 431.708 ± 10 ppm, corresponding to the doubly protonated precursors of the tryptic peptides MVDVGGQR and MFDVGGER ($G\alpha_q$-$G\alpha_s$: aa 203–210) of $G\alpha_q$-$G\alpha_s$. The 1-Da shift demonstrates the deamidation of Gln-209 to Glu. *E*) Endogenous $G\alpha_s$ was immunoprecipitated from PMT-treated MEF (1 nM, 18 h) with a G_s antibody against the C-terminal region. Immunoblot shows $G\alpha_s$ after immunoprecipitation with anti-G_s antibody. *F*) Combined extracted ion chromatogram (Q-TOF data) for *m/z* 452.464 ± 20 ppm corresponding to the quadruple protonated precursors of the tryptic peptide VNFHMFDVGGQRDER ($G\alpha_s$: aa 203–217) of $G\alpha_s$. No deamidation of Gln-213 was detectable.

S6). Tryptic digestion and LC-MS/MS analysis confirmed PMT-mediated deamidation of the target Gln in $G\alpha_{i2}^{\Delta HD}$, as evidenced by detection of the deamidated peptide (199-MFDVGGER-206; *m/z* 455.708^{2+}) *vs.* the unaffected peptide (199-MFDVGGQR-206; *m/z* 455.216^{2+}). In the case of $G\alpha_{i2}^{swII}$, only the unaffected peptide (199-MFDVGGQR-206; *m/z* 455.216^{2+}) was detected; no deamidation product was detected.

DISCUSSION

Here, we studied the substrate specificity of PMT for α subunits of heterotrimeric G proteins. To analyze which of the G_i family members $G\alpha_{i1/2/3}$ serve as substrate of PMT, we utilized the previously described coexpression system of the G-protein α subunit and PMT in *E. coli* (27). In addition, toxin-catalyzed deamidation of the $G\alpha_i$ family members $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$ was observed after overexpression in HEK-293 cells.

MS analysis data indicate that $G\alpha_q$ and $G\alpha_{11}$ are substrates of PMT. With the use of $G_{q/11}$ gene-deletion models, it has been suggested that PMT activates $G\alpha_q$ but not $G\alpha_{11}$ (10, 16). Recent studies by the laboratory of Horiguchi (29) presented the first evidence that $G\alpha_{11}$ might also be a target of PMT. In this study, an antibody specific for the deamidated form of Glu-209 of $G\alpha_{q/11}$ was used, but no MS analysis of $G\alpha_{11}$ was offered. Previous studies, which identified $G\alpha_q$ but not $G\alpha_{11}$ as a target of PMT, were mainly based on activation of PLCβ by the G proteins. One possible explanation for the observed discrepancy could be a difference in the interaction potential of the deamidated/activated forms of $G\alpha_q$ *vs.* $G\alpha_{11}$ with the downstream effector PLCβ. In line with this model is the finding of Kamitani *et al.* (29) that PMT-induced $G\alpha_{11}$-dependent activation of PLCβ was markedly lower than the $G\alpha_q$-dependent activation of PLCβ.

In intact cells, PMT is a potent activator of Rho proteins (10, 18). It is well known that the activity of the small GTPase RhoA is regulated by heterotrimeric G proteins of the $G_{12/13}$ family (39). In the case of G_{12}, LARG is stimulated to catalyze the GDP-GTP exchange of RhoA (40). G_{13} binds p115RhoGEF, which then activates RhoA (41). Studies with $G\alpha_{12/13}$-deficient MEFs showed the involvement of $G_{q/11}$ in RhoA activa-

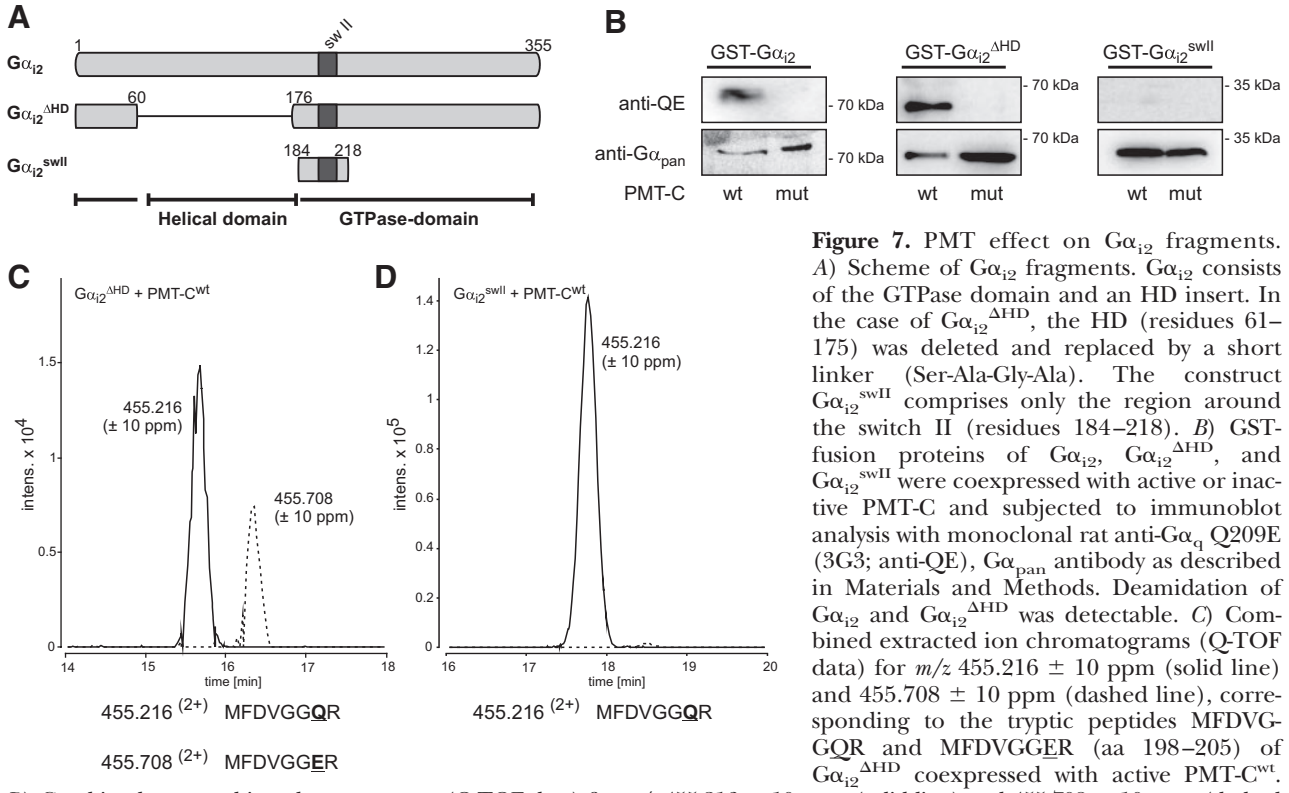

Figure 7. PMT effect on $G\alpha_{i2}$ fragments. *A*) Scheme of $G\alpha_{i2}$ fragments. $G\alpha_{i2}$ consists of the GTPase domain and an HD insert. In the case of $G\alpha_{i2}^{\Delta HD}$, the HD (residues 61–175) was deleted and replaced by a short linker (Ser-Ala-Gly-Ala). The construct $G\alpha_{i2}^{swII}$ comprises only the region around the switch II (residues 184–218). *B*) GST-fusion proteins of $G\alpha_{i2}$, $G\alpha_{i2}^{\Delta HD}$, and $G\alpha_{i2}^{swII}$ were coexpressed with active or inactive PMT-C and subjected to immunoblot analysis with monoclonal rat anti-$G\alpha_q$ Q209E (3G3; anti-QE), $G\alpha_{pan}$ antibody as described in Materials and Methods. Deamidation of $G\alpha_{i2}$ and $G\alpha_{i2}^{\Delta HD}$ was detectable. *C*) Combined extracted ion chromatograms (Q-TOF data) for *m/z* 455.216 ± 10 ppm (solid line) and 455.708 ± 10 ppm (dashed line), corresponding to the tryptic peptides MFDVGGQR and MFDVGGER (aa 198–205) of $G\alpha_{i2}^{\Delta HD}$ coexpressed with active PMT-C^{wt}. *D*) Combined extracted ion chromatograms (Q-TOF data) for *m/z* 455.216 ± 10 ppm (solid line) and 455.708 ± 10 ppm (dashed line) of $G\alpha_{i2}^{swII}$ coexpressed with active PMT-C^{wt}. Only the tryptic peptide MFDVGGQR (*m/z* 455.216), but no MFDVGGER (*m/z* 455.708), was identified.

tion (32) and that p63RhoGEF activates RhoA exclusively *via* $G_{q/11}$ (36).

PMT induces RhoA activation *via* both families of heterotrimeric G proteins. However, it was not clear whether PMT activated both members of the $G\alpha_{12/13}$ family. Here we show by immunoprecipitation of ectopically expressed $G\alpha_{12}$ or flag-tagged $G\alpha_{13}$ and subsequent LC-MS/MS analysis that PMT induced deamidation of the essential Gln residue in $G\alpha_{12}$ (Glu-231) and $G\alpha_{13}$ (Glu-226). Therefore, PMT targets both members of the $G\alpha_{12/13}$ family.

The observed substrate specificity of PMT prompted us to study additional structural determinants of PMT-targeted Gα subunits. An alignment of responsive ($G_{i1/2/3}$, G_q, $G\alpha_{11}$, G_{13}, and G_{12}), nonresponsive (G_s), and other G-protein α subunits (Fig. 5*A*) revealed high amino acid sequence identity within the switch II regions, with only minor changes present in some of the targets. The region upstream of the essential Gln (209 in $G\alpha_q$) that is modified by the toxin is nearly identical among the α subunits. In contrast, differences occur at some downstream positions; most notably, the conserved Ser at position +2 is an Asp in the unresponsive $G\alpha_{s/olf}$ family, and a conserved Glu at position +3 is a Gln in the responsive $G\alpha_{12}$. However, MS data of the respective mutants revealed that these residues are not sufficient to define substrate recognition by PMT.

Moreover, we studied whether parts of G proteins, which are responsible for direct interaction with their specific effectors, are crucial for PMT activation. We observed that a chimera, consisting of the first 216 amino acid residues of $G\alpha_q$ fused to the C terminus of $G\alpha_s$ (residues 221–380), was activated by PMT. These results indicate that the G-protein effector-binding region is not crucial for PMT action. The intensity of PMT-deamidated $G\alpha_q$-$G\alpha_s$ in MS analysis is remarkably lower compared with other tested PMT substrates. However, utilized MS techniques do not allow quantification. Therefore, no conclusion can be drawn regarding the effectiveness of deamidation. Based on the interaction sites of G_s with adenylyl cyclase, which mainly encompass the α3-β5 loop of G_s and the switch II region (42), our data suggest that the effector interaction site downstream of the switch II region is not essentially involved in defining substrate specificity of PMT. These findings might offer the possibility to engineer G proteins of specific functions (*e.g.*, for substitution of genetic malfunction), which are deamidated and constitutively activated by PMT.

To clarify the involvement of the HD in substrate recognition and/or specificity, we deleted this domain from $G\alpha_{i2}$ ($G\alpha_{i2}^{\Delta HD}$). The construct was created in analogy to studies with $G\alpha_s$ on the structure/function relationship of the Ras-like GTPase domain and the HD (43). The remaining Ras-like GTPase domain of $G\alpha_{i2}$ still harbors the essential Gln residue in the switch II region. Coexpression with active PMT, followed by immunoblot and LC-MS/MS analysis, revealed that the toxin also accepts this deletion mutant as a substrate. In contrast, another mutant of $G\alpha_{i2}$, consisting of a small region around switch II itself ($G\alpha_{i2}^{swII}$, residues 184–218), was

not deamidated by PMT. These results indicate that the HD is not essential for substrate recognition.

Recently, the structure of the active state ternary complex comprised of agonist occupied β-adrenoceptor and G_s was reported (44). Interestingly, this nucleotide-free structure showed a strong displacement of the HD relative to the Ras-like GTPase domain. Westfield *et al.* (45) proposed that the reorientation of the HD of $G\alpha_s$ facilitates accessibility to the catalytic Arg-201 for ADP-ribosylation by cholera toxin. In addition, the catalytic Gln residue of $G\alpha_s$ is particularly exposed in the nucleotide-free $G\alpha_s$. There is reasonable evidence (46) that other family members of the heterotrimeric G proteins, including the PMT targets $G\alpha_i$ and $G\alpha_{q/11}$, show similar structural changes during nucleotide exchange. The nucleotide-free state with a reoriented HD could present a PMT-accessible switch II Gln residue. Further studies will be needed to clarify whether this nucleotide-free structure of the heterotrimeric complex is the preferred target structure for PMT action.

The relevance of the HD for PMT deamidation should also be reinvestigated with respect to the new structural findings. Alignments of the α subunits of heterotrimeric G proteins show a high similarity in their GTPase domains yet striking differences in the HDs (47). The result that the HD of the PMT target $G\alpha_{i2}$ is not necessary for toxin recognition does not exclude a possible negative effect of the HD in the nonresponsive $G\alpha_s$. In future studies, we will test whether the HD of $G\alpha_s$ is an inhibitory element in the $G\alpha_s$ subunit for PMT-induced deamidation.

Taken together, the studies reported herein extend our knowledge of the substrate specificity of PMT. The toxin deamidates the G_i family members $G\alpha_{i1/2/3}$. Furthermore, $G\alpha_q$ and $G\alpha_{11}$ and both members of the $G_{12/13}$ family are deamidated. All these deamidation reactions, catalyzed by PMT, cause persistent activation of the G proteins. Mutations of the crucial Gln residue of Gα subunits, which is involved in GTP hydrolysis, are known to have transforming capability. This is true for $G\alpha_i$, presumably through activation of the mitogen-activated protein kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) signaling pathway (48). Because this same Gln of $G\alpha_i$ is a target of PMT and the same pathway is stimulated by PMT (9), it is possible that this contributes to the transforming potential of PMT. Recently, somatic mutations of *GNAQ* or *GNA11*, the genes encoding for $G\alpha_q$ and $G\alpha_{11}$, were found in uveal melanoma and blue naevi (49, 50). Again, the identical Gln-209 is affected, which is deamidated by PMT. Moreover, GTPase-deficient mutants of $G\alpha_{12}$ or $G\alpha_{13}$ were described as most powerful transforming Gα subunits (51).

Usually, all these mutants occur at the crucial sites of the switch II Gln, but also at the essential Arg in the switch I region (48, 51). In contrast to G-protein-coupled receptor-induced effects, which occur fast and transiently, GTPase-deficient mutants signal continuously. Therefore, tightly regulated cell functions become deregulated and can contribute to the hallmarks of cancer, such as sustained proliferative signaling, resisting cell death or metastasis (13, 52).

It is fascinating that PMT affects heterotrimeric G proteins at the identical site where oncogenic mutations were identified. Although PMT does not induce gene mutations, deamidation of the crucial Gln in the switch II is long lasting and is apparently terminated only by G-protein degradation (Fig. 2*B*). Consequently, PMT is already discussed as a potential carcinogen (15). FJ

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