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Serine 331 is major site of phosphorylation and desensitization induced by protein kinase C in thromboxane receptor α[1]

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ABSTRACT

AIM: To identify the specific serine/threonine residues in the C-terminal tail of thromboxane receptor α (TPα) being phosphorylated and desensitized, and various alanine mutants of these serine/threonine residues were checked for their ability to serve as substrates. **METHODS:** To facilitate the identification of the intracellular domains involved in phosphorylation, glutathione S-transferase (GST)-intracellular domain fusion proteins were used as substrates for the purified PKC, and then the cDNA of phosphorylated protein was mutagenized to localize the major site of receptor phosphorylation induced by protein kinase C. Human embryonic kidney (HEK) 293 cells stably transfected with the His-tagged wild type or mutant TPα were used to study the phosphorylation and desensitization. **RESULTS:** Only the C-terminal tail can be used as a substrate for the purified PKC. Ser-331 (mP4) was demonstrated to be heavily phosphorylated, Ser-324 (mP1) was shown to be slightly phosphorylated, Ser-329 was illustrated to be faintly phosphorylated, and other Ser/Thr residues were not found to be phosphorylated. Phorbol-12-myristate-13-acetate (PMA) induced receptor phosphorylation in HEK 293 cells expressing the wild type TPα. However, PMA did not significantly trigger receptor phosphorylation in HEK 293 cells expressing the S331A mutant receptor. Pretreatment of the cells expressing the wild type with PMA inhibited I-BOP induced Ca^{2+} release, however, pretreatment of the cells expressing the S331A mutant receptor with PMA did not abolish I-BOP induced Ca^{2+} release . **CONCLUSION:** Ser-331 is the major and crucial site of receptor phosphorylation and desensitization.

INTRODUCTION

Thromboxane A_2 (TXA_2) is a lipid mediator with potent platelet aggregatory and vascular and airway smooth muscle constrictive activities[1,2]. TXA_2 is also involved in mitogenesis of vascular smooth muscle cells[3], and apoptosis of immature thymocytes[4]. Its effects are mediated by specific TXA_2 receptors (TP) and subjected to regulatory control[5]. There are two isoforms of the TP, α and β, which are identical for their first 328 amino acids and differ exclusively in their C-terminal tails as a result of alternative splicing from a single gene[6,7]. The TP belongs to heptahelical G-

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protein coupled receptor (GPCR) superfamily. Receptors of this superfamily feature 7 transmembrane domains and three each of the intracellular and the extracellular domains as well as a N-terminal and a C-terminal tail. The GPCR are known to undergo receptor desensitization following agonist stimulation[8,9]. It involves uncoupling of G-proteins from a receptor, sequestration of receptor from cell surface, and down regulation of total receptor number[10]. Receptor phosphorylation induced by agonist stimulation is thought to contribute to each of these processes[8]. Agents that promote cross-talk between receptors can also induce receptor phosphorylation. Receptor phosphorylation is mediated by receptor-specific kinases and general kinase systems such as PKA and PKC. These kinases systems provide a mechanism for regulating receptor activity through negative feedback loops (homologous desensitization) as well as cross-talk between different receptor systems (heterologous desensitization)[11]. The involvement of G protein-coupled receptor kinase (GRK) in the TP phosphorylation and desensitization[12] and in the TP internalization[13] was recently demonstrated. Participation of the second messenger kinases in the TP phosphorylation and desensitization has also been report-ed[11,14-16].

However, the precise localization of the sites of phosphorylation mediated by PKC remains to be determined. In this report, we examine the phosphorylation and desensitization of the human TPα overexpressed in human embryonic kidney (HEK) 293 cells induced by phorbol-12-myristate-13-acetate (PMA) and identify Serine (Ser) -331 as the major site of phosphorylation induced by PMA.

MATERIALS AND METHODS

Materials The pcDNA3 expression vector was from Invitrogen (Carlsbad, CA, USA). pGEX-2T plasmid vector was from Pharmacia Biotech (Piscataway, NJ, USA). T4 DNA ligase and *Vent* DNA polymerase were from New England Biolabs, Inc (Beverly, MA, USA). *pfu* DNA polymerase was from Stratagene (La Jolla, CA, USA). Human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection (Manassas, VA, USA). *Taq* DNA polymerase, *Eco*R I, *Bam*H I, heat inactivated fetal bovine serum, antibiotic-antimycotic, and geneticin selective antibiotic (G418) were from GIBCO-BRL (Rockville, MD, USA). The QIAprep Spin Plasmid Miniprep Kit, QIAquick PCR Purification Kit, QIA Quick Gel Extraction Kit, and Effectene Transfection Reagent were from QIAGEN (Valencia, CA, USA). I-BOP was from Cayman Chemical (Ann Arbor, MI, USA). ^{32}Pi (18.5 TBq/L) and [γ-^{32}P] ATP (370 TBq mol/L) were from ICN Pharmaceuticals, Inc (Costa Mesa, CA, USA). Fura-2/AM and recombinant bovine protein kinase C α were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA, USA). Horseradish peroxidase (HRP)-linked protein A was obtained from Transduction Laboratories (Franklin Lakes, NJ, USA). Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Phorbol-12-myristate-13-acetate (PMA), Dulbecco's Modified Eagle's Medium (DMEM), Ni^{2+}-NTA agarose, GST agarose, phenylmethylsulfonyl fluoride (PMSF), leupeptin, soybean trypsin inhibitor, benzamidine, and other chemicals were from Sigma (St Louis, MO, USA). Electro-chemiluminescence (ECL^+) plus Western blotting detection system was from Amersham (Piscataway, NJ, USA). Rabbit antiserum against N-terminal sequence (MWPNGSSLGPCFRPTN) of human TPα was prepared as previously described[12].

Subcloning of the C-terminal His-tagged human TPa cDNA His-tagged human TPα cDNA was amplified from pBacPak8-TPα plasmid DNA by PCR using *Vent* DNA polymerase as previously described[17]. The PCR product was subcloned into the mammalian expression vector pcDNA3 at *Bam*H I and *Eco*R I sites. The insertion of the TP-$(His)_6$ cDNA was confirmed by DNA sequencing.

Site-directed mutagenesis of the TPa and its C-terminal tail Site-directed mutagenesis was carried out by the Megaprimer method[18]. Both His-tagged S331A mutant of the TPα and various mutants of C-terminal tail were prepared in this manner and cloned into pcDNA3 and PGEX-2T expression vector, respectively.

Western blot analysis The cell membranes (50 μg) prepared from HEK293 cells expressing the His-tagged wild type and mutant TPα were subjected to SDS-PAGE on 10 % polyacrylamide gel. The proteins were then electrophoretically transferred onto PVDF membranes. All other methods were carried out as previously described[12,17].

Expression of the intracellular (IL) domain specific peptides of the TPa as glutathione S-transferase (GST)-fusion proteins The DNA sequences encoding the three IL loops and C-tail were generated

by PCR using *Taq* DNA polymerase and pBacPak8 TPα plasmid DNA as template. The oligonucleotide primers and conditions of PCR amplification, recombination of pGEX-2T plasmid DNA and IL domain cDNA, and expression of the IL domain specific peptides of the TPα were carried out as previously described[17]. Several colonies were screened for the relative level of expression GST fusion by the 1-chloro-2,4-dinitrobenzene (CDNB) assay[19]. The GST fusion proteins were purified using GST agarose affinity chromatography as previously described[20].

***In vitro* phosphorylation of the intracellular domains of the TP**a *In vitro* phosphorylation of the intracellular domain specific peptides of the TPα was performed as previously described[17, 21]. Only modification was that 1000 U purified PKC replaced GRK.

Expression of the His-tagged human wild type and mutant TPa **in HEK 293 cells** HEK 293 cells were grown in 90 % DMEM supplemented with 10 % heat-inactivated fetal bovine serum (FBS), gentamicin, and antibiotic-antimycotic at 37 ℃ in a humidified atmosphere of 95 % air and 5 % CO_2. Cells were subcultured every 3 d after becoming confluent by use of 0.25 % trypsin with edetic acid 1 mmol/L and plated at a density of 1×10^8/L.

To create cell lines stably expressing the wild type and the mutant TPα, pcDNA3 expression vector containing the cDNAs of the wild type and the mutant His-tagged TPα, were respectively transfected into HEK 293 cells using Effectene Transfection Reagent according to the manufacturer directions. Briefly, 5×10^5 cells were plated per 60 mm dish in 5 mL of completed medium the day before transfection. One μg of DNA was first mixed with DNA-condensation buffer to a total volume of 150 μL, then 8 μL of Enhancer was added. After incubation at 25 ℃ for 5 min, 25 μL of Effectene Transfection Reagent was added to the DNA-enhancer mixture, mixed, and incubated at 25 ℃. After 15 min, 1 mL of cell culture medium was added to the transfection complexes. This solution was mixed and added to 60 mm dishes containing washed HEK 293 cells. Cells were incubated with the complexes at 37 ℃ and 5 % CO_2 for 2 d to allow for gene expression. To isolate permanent transfectants, G418-resistant cells were selected in complete culture medium containing 500 mg/L G418 as previously described[22]. Expression of the wild type and the mutant TPα was monitored by the Western blotting analysis using a rabbit antiserum against the N-terminal sequence of the TPα as previously described[12].

***In vivo* phosphorylation of the wild type and the mutant TP**a *In vivo* phosphorylation of the wild type and the mutant TPα was carried out as previously described[17] with minor modification, which is that PMA (100 nmol/L) replaced U46619 (1 μmol/L) or I-BOP (100 nmol/L).

Measurement of intracellular calcium release Intracellular calcium release was measured by fluorescence excitation of cells loaded with the fluorescent probe Fura-2/AM as previously described[23] with minor modifications. Briefly, the HEK 293 cells stably transfected with TPα or mutant were collected and washed with Fura-2 assay buffer (Na_2HPO_4 4.3 mmol/L, NaH_2PO_4 24.3 mmol/L, K_2HPO_4 4.3 mmol/L, pH 6.8, NaCl 113 mmol/L, and *D*-glucose 5 mmol/L). The cells were resuspended in the loading buffer (Fura-2 assay buffer added $CaCl_2$ 1 mmol/L, Fura-2/AM 10 μmol/L and 0.5 % BSA) at a concentration of 1×10^9/L. The suspension was incubated at 37 ℃ for 1 h, pelleted by centrifugation at 1000×*g* for 10 min, washed with the Fura-2 assay buffer twice. The cells were resuspended in the Fura-2 assay buffer (added PMA 100 nmol/L or vehicle) at a concentration of 1×10^9/L and incubated at 37 ℃ for 10 min, pelleted by centrifugation at 1000×*g* for 10 min, washed with the Fura-2 assay buffer twice again. The pretreated cells were resuspended at approximately 5×10^9/L in the Fura-2 assay buffer (added $CaCl_2$ 1 mmol/L). The Ca^{2+} signal induced by the TPα agonist I-BOP was examined by Fura-2 fluorescence in a Hitachi F-2000 Fluorescence Spectrophotometer at 37 ℃ with excitation and emission wavelengths at 340 and 510 nm, respectively. The reaction was initiated by adding I-BOP to a final concentration of 100 nmol/L.

Statistic analysis Results were presented as mean±SD. For statistical analysis, *t* test was used. Differences were considered significant at a level of $P<0.05$.

RESULTS

***In vitro* phosphorylation of PKC on the intracellular domains of the TP**a **and C-terminal mutants** To localize the sites of phosphorylation among the intracellular domains, GST-IL fusion proteins were used as substrates for the purified PKC. Only the C-terminal tail could be used as a substrate for the purified PKC (Fig 1). There were 6 Ser/Thr residues (Ser-324, Thr-

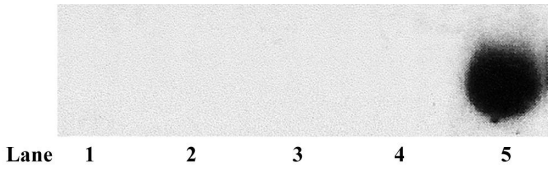

Fig 1. *In vitro* phosphorylation of the cytoplasmic domains of TPa by the purified PKC. Two mg of GST-intracellular loop fusion protein was used as a substrate for the purified PKC (1000 U). 1) GST; 2) GST-1st intracellular loop; 3) GST-2nd intracellular loop; 4) GST-3rd intracellular loop; 5) GST-C-terminal tail. A representative experiment of three qualitatively similar results was shown.

325, Ser-329, Ser-331, Thr-337, and Ser-340) in the C-terminal tail of the TPα. Two of these 6 residues appeared to locate closer to each other as a group. Mutation of 2 of these residues to alanines (mC3, mC4, and mC5) followed by mutation of 4 of these residues (mC6, mC7, and mC8) and all of these 6 residues (mC9) were carried out as shown in Fig 2. Among these C-terminal tail mutants, the mutants (mC3, mC5, and mC7) having unaltered Ser329/Ser331 were found to be heavily phosphorylated and the mutants (mC4 and mC8) having unaltered Ser324/Thr325 were found to be slightly phosphorylated (Fig 2). To narrow down the site of phosphorylation and confirm the above finding, mutants (mP1 to mP4) having a single Ser/Thr site to be unaltered were generated and used as substrates for the purified PKC again. The mC6 was served as negative control and checked up again. Ser-331 (mP4) was demonstrated to be heavily phosphorylated, Ser-324 (mP1) was shown to be slightly phosphorylated and Ser-329 (mP3) was shown to be faintly phosphorylated. Other Ser/Thr residues were not found to be phospho-

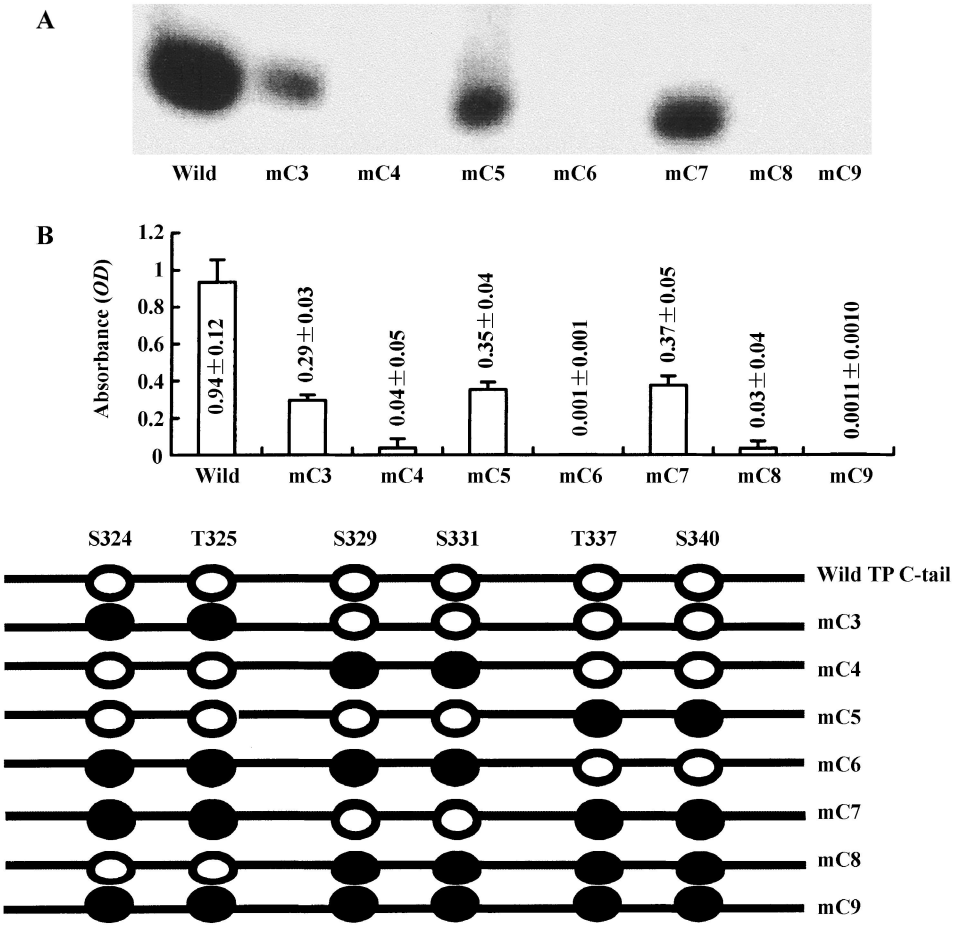

Fig 2. *In vitro* phosphorylation of the various double and multiple mutants of the C-terminal tail of the TPa. A) The dark circle indicates the specific amino acid residue being mutated to an alanine. Two mg each of the GST-C-terminal tail mutant fusion protein was used as a substrate for purified PKC as described in Fig 1. A representative experiment of three qualitatively similar results was shown. B) Densitometric analysis of the phosphorylation extents were carried out in 3 qualitatively similar results. *n*=3 independent experiments. Mean±SD.

rylated (Fig 3).

***In vivo* PMA-inducing phosphorylation of PKC on the wild type and the mutant TP**a To examine if the receptor was phosphorylated and the phosphorylated receptor contributed to subsequent desensitization, HEK 293 cells expressing the TPα-$(His)_6$ were treated with PMA and the phosphorylated receptor was isolated by Ni^{2+}-NTA agarose and subjected to SDS-PAGE. PMA made the receptor phosphorylate in a dose-dependent manner (Fig 4). To verify that Ser-331 was the potential site of receptor phosphorylation induced by PMA in intact cells expressing the TPα receptor, HEK 293 cells stably transfected with the pcDNA3-TPα-$(His)_6$ having S331A mutation were generated and used for *in vivo* phosphorylation studies. PMA induced receptor phosphorylation in HEK 293 cells expressing the wild type TPα as shown earlier. However, this agent did not significantly trigger receptor phosphorylation in HEK 293 cells expressing the S331A mutant receptor (Fig 5). The lack of phosphorylation in mutant receptor was not due to an insufficient expression of the receptor. A comparable receptor expression was observed in the wild type and the mutant receptor as shown by Western blot analysis (Fig 6).

***In vivo* PMA-inducing desensitization of PKC on the wild type and the mutant TP**a Whether or not the mutant receptor still underwent desensitization remained to be determined. HEK 293 cells expressing the wild type receptor or the S331A mutant receptor were treated with PMA before stimulation with TP agonist I-BOP. Pretreatment of cells expressing the wild type receptor with PMA inhibited I-BOP induced Ca^{2+} release. However, pretreatment of the cells expressing the S331A mutant receptor with vehicle augmented

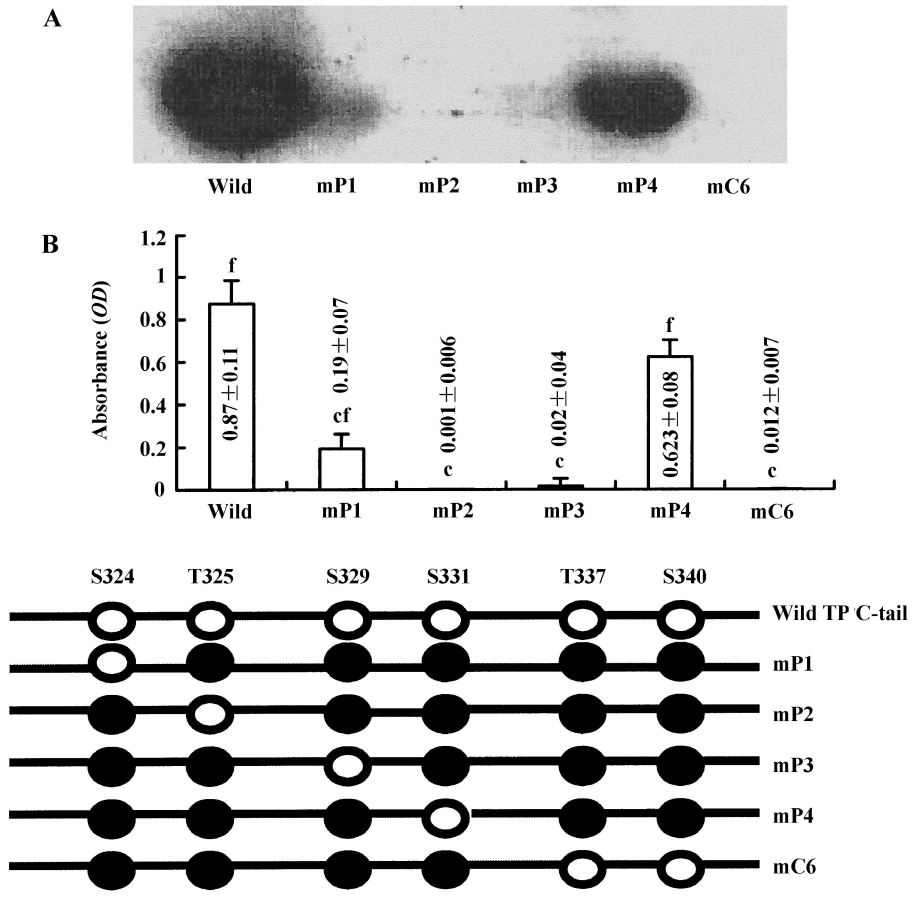

Fig 3. ***In vitro*** **phosphorylation of the various multiple sites mutants of the C-terminal tail of the TP**a. **A) The reaction conditions and the SDS-PAGE analysis of GST fusion proteins were carried out as described in Fig 1 and 2. A representative experiment of three qualitatively similar results was shown. B) Densitometric analysis of the phosphorylation extents was carried out in 3 qualitatively similar results. *n*=3 independent experiments. Mean±SD. $^{c}P<0.01$ *vs* PKC-induced phosphorylation of wild type C-terminal tail. $^{f}P<0.01$ *vs* PKC-induced phosphorylation of mutant mC6.**

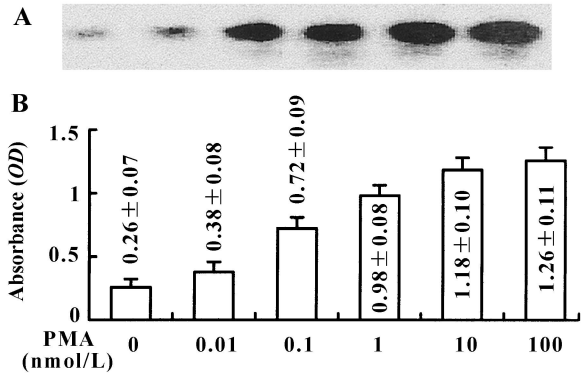

Fig 4. Dose dependent phosphorylation of receptor induced by PMA in HEK293 cells overexpressing the TPa. A) Cells (1×10^9/L) stably transfected with pcDNA-TPa-$(His)_6$ were metabolically labeled with ^{32}P and then respectively treated with increasing concentrations of PMA for 10 min as indicated. B) Densitometric analysis of the phosphorylation extents was carried out in 3 qualitatively similar results. *n*=3 independent experiments. Mean±SD.

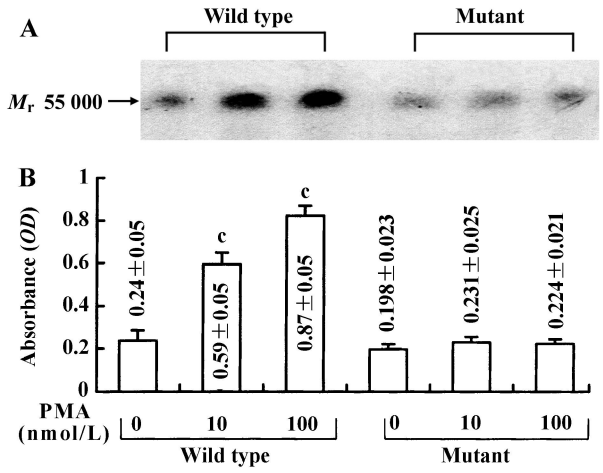

Fig 5. *In vivo* phosphorylation of the wild type and the S331A mutant receptor induced by PMA in HEK293 cells overexpressing the TPa. A) Cells (1×10^9/L) expressing the wild type or the S331A mutant receptor were metabolically labeled with ^{32}P and then respectively treated with PMA at the indicated concentrations. A representative experiment of three qualitatively similar results was shown. B) Densitometric analysis of the phosphorylation extents was carried out in 3 qualitatively similar results. *n*=3 independent experiments. Mean±SD. $^cP<0.01$ *vs* 0 nmol/L PMA-induced phosphorylation of wild type C-terminal tail.

I-BOP induced Ca^{2+} release as compared to that of the cells expressing the wild type receptor (Fig 7).

DISCUSSION

Activation of intracellular protein kinases by ex-

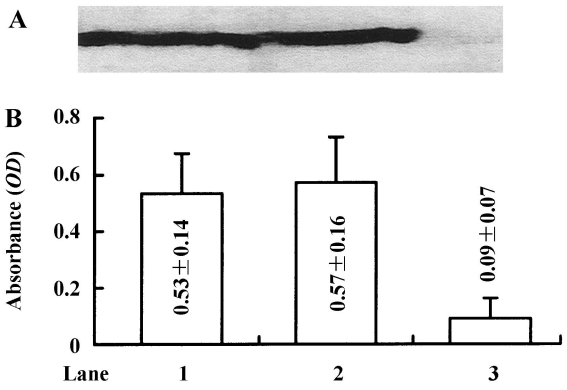

Fig 6. Western blotting assay of the wild type and mutant TPa stably expressed in HEK293 cells. A) The cell membranes (100 mg) prepared from cells stably transfected with pcDNA3 encoding the wild type receptor (lane 1), the mutant receptor (lane 2), or the pcDNA3 vector alone (lane 3) were respectively subjected to 10 % SDS-PAGE. A representative experiment of 3 qualitatively similar results was shown. B) Densitometric analysis of the immunoreactive bands was carried out in 3 qualitatively similar results.

ogenous agonists or accelerant generally leads to phosphorylation of the intracellular domains and the subsequent desensitization of the receptors. The putative third intracellular loop and the C-terminal tail have been highlighted as the most important structural features involved in agonist-induced phosphorylation and desensitization of the GPCR[24]. Recent studies on the TP have confirmed that the C-terminal tail appears to play an important role in phosphorylation, desensitization, and internalization. Spurney and Coffman[11] first demonstrated that the C-terminus of the TP contributes to coupling and desensitization in a mouse mesangial cell line. Subsequently, Spurney[15] showed that the C-terminal tail of the mouse TP was involved not only in agonist but also phorbol ester induced phosphorylation and desensitization. The S321A/S322A/S328A (equivalent to Ser-324, Ser-325 and Ser-331 in humans) mutant receptor was found to be attenuated as compared to the wild type in phosphorylation and desensitization induced by agonist U-46619 and by a phorbol ester. Recently, we also reported that the C-terminal tail but not the other intracellular loops of the human TPα was an excellent substrate for GRK *in vitro*[17].

To facilitate the identification of a specific intracellular domain(s) involved in the PKC mediated phosphorylation, we had constructed the GST-fusion protein expression system to generate GST-IL fusion

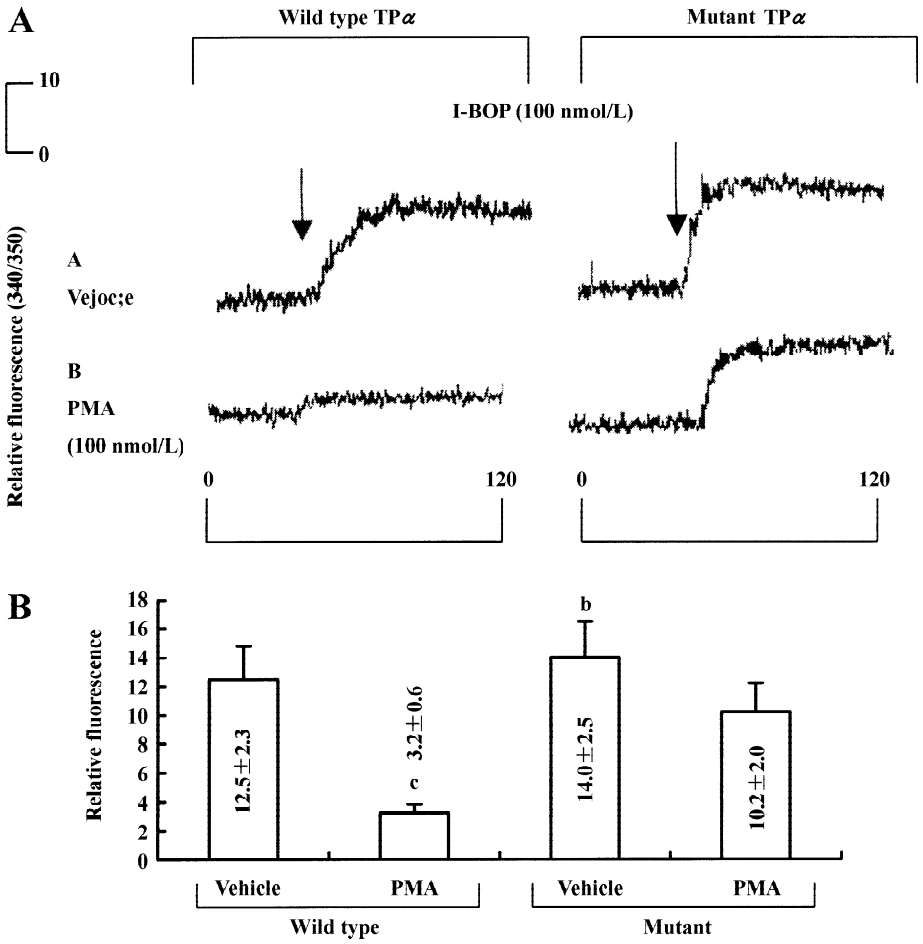

Fig 7. Desensitization of the Ca^{2+} response induced by PMA in cells overexpressing the wild type or the S331A mutant TPa. **A) Cells (1×10^9/L) expressing comparable amount of the wild type or the S331A mutant receptor were treated with PMA for 10 min before the Ca^{2+} mobilization was initiated by 100 nmol/L I-BOP for 2 min. A representative experiment of 3 qualitatively similar results was shown. B) Analysis of relative fluorescence on $[Ca^{2+}]_i$ mobilization was carried out in three qualitatively similar results. *n*=3 independent experiments. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* I-BOP-induced $[Ca^{2+}]_i$ mobilization of wild type receptor with vehicle.**

proteins and their mutants as potential substrates for PKC. It appeared that only the C-terminal tail fusion protein could be used as a substrate for the PKC. Similar conclusion was reached by Wang *et al*[25]. Among all the 6 Ser/Thr residues in the C-terminal tail examined, Ser-331 appeared to be the major site of phosphorylation, although Ser-324 and Ser-329 were phosphorylated to a very minor extent based on phosphorylation studies on various C-terminal tail mutants. The result is similar to role of C-terminal serines in desensitization and phosphorylation of the mouse thromboxane receptor[15]. That Ser-331 is a predominant site of phosphorylation was further supported by *in vivo* studies in which HEK 293 cells were stably transfected with the S331A mutant of the His-tagged TPα and stimulated with the PKC accelerant PMA for 10 min because PMA made PKC down-regulation for prolonged treatment[26]. Phosphorylation of the mutant receptor induced by the accelerant was not apparent while the wild type receptor was phosphorylated significantly as expected. This was further supported by the desensitization study in which HEK 293 cells expressing the S331A mutant of the TPa still responded to the agonist, I-BOP, in releasing Ca^{2+} although attenuated after treatment with PMA. The wild type receptor was barely responsive to the agonist in the Ca^{2+} mobilization after treatment with PMA. Obviously, the phosphorylation of Ser-331 was responsible for the effect of the PMA. Attenuation of the I-BOP induced Ca^{2+} response in treated cells as compared to the untreated cells expressing mutant receptor was probably in part due to minor phosphorylation at the other two serine residues observed *in vitro* studies. The fact that the I-BOP induced Ca^{2+} response in cells expressing the mutant receptor was faster than that in

cells expressing the wild type receptor also suggests that Ser-331 may be one of the sites involved in PMA induced receptor phosphorylation. Inability to phosphorylate the S331A mutant receptor induced by PMA appeared to decrease the effect of phosphorylation and subsequently desensitization induced by the agent. Consequently, Faster Ca^{2+} response was observed with the mutant receptor.

Our studies first found that C-terminal serines (324, 329, and 331) of human TPα were responsible for PKC-inducing or PMA-activating PKC phosphorylation of TPα and the subsequent desensitization of the receptors. Among the serines, Ser-331 is the major and crucial site of receptor phosphorylation and desensitization.

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