The Protein Kinase C-dependent Phosphorylation of Serine 166 Is Controlled by the Phospholipid Species Bound to the Phosphatidylinositol Transfer Protein α*

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The charge isomers of bovine brain PI-TPα (i.e. PI-TPα containing a phosphatidylinositol (PI) molecule and PI-TPαII containing a phosphatidylcholine (PC) molecule) were phosphorylated in vitro by rat brain protein kinase C (PKC) at different rates. From the double-reciprocal plot, it was estimated that the Vmax values for PI-TPα and II were 2.0 and 6.0 nmol/min, respectively; the Ks values for both charge isomers were about equal, i.e. 0.7 μM. Phosphorylation of charge isomers of recombinant mouse PI-TPα confirmed that the PC-containing isomer was the better substrate. Phosphoamino acid analysis of in vitro and in vivo 32P-labeled PI-TPα showed that serine was the major site of phosphorylation. Degradation of 32P-labeled PI-TPα by cyanogen bromide followed by high pressure liquid chromatography and sequence analysis yielded one 32P-labeled peptide (amino acids 104–190). This peptide contained Ser-166, Ser-175, Ser-182, Ser-188, Ser-193, and the consensus PKC phosphorylation site Ser-166. Replacement of Ser-166 with an alanine did not affect PI-TPα activity; instead, the PKC phosphorylation of PI-TPα(S166A) and Myc-tagged PI-TPα(S166D) were not affected by phorbol ester or platelet-derived growth factor induced the rapid relocation of PI-TPα to perinuclear Golgi structures concomitant with a 2–3-fold increase in lysophosphatidylinositol levels. This relocation was also observed for Myc-tagged wtPI-TPα expressed in NIH3T3 cells. In contrast, the distribution of Myc-tagged PI-TPα(S166A) and Myc-tagged PI-TPα(S166D) were not affected by phorbol ester, suggesting that phosphorylation of Ser-166 was a prerequisite for the relocation to the Golgi. A model is proposed in which the PKC-dependent phosphorylation of PI-TPα is linked to the degradation of PI-TPα.

Phosphatidylinositol transfer protein (PI-TP)1 is a ubiquitously protein that has been shown to play an essential role in secretion (vesicle flow) and in phospholipase C-dependent signaling as was established in reconstituted systems (1–4). In addition, PI-TP may have a role in delivering substrate to the phosphatidylinositol 3-kinase complex (5, 6). Two isoforms of PI-TP have been identified, PI-TPα and PI-TPβ (7–9). Both isoforms transfer phosphatidylinositol (PI) and phosphatidylcholine (PC) between membranes in vitro (10). PI-TPβ expresses an additional activity for sphingomyelin (7, 11). In studies with the reconstituted systems, PI-TPα and β behaved similarly. Recently, PI-TPα overexpressed in NIH3T3 cells was shown to enhance the constitutive levels of lysophosphatidylinositol (lysoPI) (12). Overexpression of PI-TPα in these cells had no effect on lysoPI formation but stimulated the conversion of ceramide into sphingomyelin (13). In addition to these soluble PI-TPs, a membrane-bound form of PI-TP was detected containing a PI-TPα homology domain at the N terminus (amino acids 1–257) and six putative membrane-spanning domains. This retinal degeneration B (RdgB) protein was originally identified in Drosophila (14).

Localization studies by indirect immunofluorescence and by microinjection of fluorescently labeled PI-TPα have shown that PI-TPα is mainly localized in the nucleus and in the cytosol and that PI-TPβ is mainly associated with the Golgi membranes (7, 8). Upon stimulation of Swiss mouse 3T3 fibroblasts by growth factors that activate the PI signaling pathway or by phorbol 12-myristate 13-acetate (PMA), PI-TPα became rapidly associated with the Golgi membranes. Under these conditions PI-TPα was found to be phosphorylated, suggesting that this modification may be a prerequisite for its association with the Golgi (15, 16). PI-TPα was also a substrate for protein kinase C (PKC) in vitro in agreement with the presence of five putative phosphorylation sites: Thr-59, Thr-169, Thr-198, Thr-251, and Ser-166 (15, 16).

Two charge isomers of PI-TPα are present in tissues and cells of which one isomer carries a PI molecule (PI-TPαI) and the other a PC molecule (PI-TPαII) (17). The cellular concentration of PI-TPαI is about 8-fold higher as compared with that of PI-TPαII. Because the affinity of PI-TPα for PI is about 16-fold lower than the affinity for PI, the relative amounts of the two isoforms reflect the accessible pools of PI and PC in the cell. To date, no comparable charge isoforms of PI-TPβ have been detected. The physiological significance of the two charge isoforms of PI-TPα is not yet known. It has been suggested that the yeast analogue of PI-TP, SEC14p, acts as a sensor of these phospholipid pools in the Golgi inasmuch as the two charge isoforms affect PI and PC metabolism differently (18). Given the ability of PI-TPα to be involved in both PI and PC metabolism.
metabolism (12, 19), one may expect a regulatory mechanism in the cell to be able to discriminate between PI-TPα and II. Because PI-TPα is a substrate for PKC, we have investigated whether the phospholipid bound to PI-TP has an effect on the in vitro phosphorylation. It will be shown that PI-TPαII is more rapidly phosphorylated by PKC than PI-TPαI. Both charge isomers have one major phosphorylation site (Ser-166), replacement of which with Ala or Asp completely abolished the transfer activity. A model will be presented in which the phosphorylation of PI-TPα is linked to the agonist-induced production of lysoPI.

**EXPERIMENTAL PROCEDURES**

*Materials—Egg yolk PC, soybean PI, phosphatidic acid, phosphatidylserine, PMA, ATP, phosphoserine, phosphothreonine, and phosphotyrosine were obtained from Sigma. The pBluescript SK*− vector and the Quickchange site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA). The oligonucleotides were synthesized by Eurogentec, Belgium. The pET-15b vector was obtained from Novagen (Madison, WI). The *Escherichia coli* strain BL21(DE3) was obtained from Dr. J. H. Veerkamp (Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands). Isopropyl-β-D-thiogalactopyranoside was purchased from Promega (Madison, WI). Ni**2+**-High Bond matrix was from Invitrogen (San Diego, CA). γ**−**ATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. Cellulose TLC plates and TPP-trypsin were purchased from Merck KGaA. The QuickGENES Transfection Reagent and the anti-c-Myc monoclonal antibody were from Roche Molecular Biochemicals.

**Purification of Protein Kinase C—PKC was purified from rat brain by a modified procedure previously described by Huang et al. (20). Rat brains (20–40 g of tissue) were homogenized, and the cytosolic fraction was subsequently purified on DEAE-Sepharose, Sephacryl 200 and phenyl-Sepharose columns. The purified enzyme has a specific activity of 200 nmol of phosphate/min/mg protein when assayed with histone IIIa as substrate. The purified enzyme is stable for several months when kept at −80 °C in 50% glycerol and 0.01% Triton X-100.

**Purification of Bovine PI-TPα and II and of Recombinant Mouse PI-TPα—**PI-TPα and II were purified from bovine brain cytosol as described by van Paridon et al. (17). The cDNA encoding mouse PI-TPα was expressed in *E. coli*, and the protein was purified as described by Geijtenbeek et al. (16).

**Preparation of Recombinant PI-TPα I and II—**The mouse recombinant PI-TPα (recPI-TPα) purified from *E. coli* contains one molecule of phosphatidylglycerol (PG) (16). To exchange the PG molecule for a PI or PC molecule, recPI-TPα (28 nmol) was incubated with PI (385 nmol) present in unilamellar vesicles consisting of PI:PC (30:70 mol %) or with PC (70:30 mol %) in vesicles of PC:phospholipids (70:30 mol %). Vesicles were prepared in 20 mM Tris buffer that was equilibrated in 20 mM NaCl by sonication under nitrogen for 10 min. Vesicles and protein were incubated for 15 min at 37 °C. The reaction was stopped by the addition of MgCl₂ to a final concentration of 5 mM. Protein and vesicles were separated on a DEAE-cellulose column that was equilibrated in 20 mM Tris/HCl buffer (pH 7.5). The protein content was determined using the Bradford procedure was carried out as described by Snoek et al. (15). Phosphorylation of PI-TPα in Vivo—Swiss mouse 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum and buffered with NaHCO₃ (44 mM) in a 7.5% CO₂ atmosphere. Near-confluent cell cultures in 75-cm² flasks were labeled for 4.5 h with 1.5 Ci of carrier-free [γ**−**32P]ATP. The Ca**2+**-phospholipid-independent phosphorylation was determined in the presence of 1 mM EDTA, and the Ca**2+**/phospholipid-dependent phosphorylation was determined in the presence of 1 mM Ca**2+**, 96 µg/ml phosphatidylserine, and 2.2 µg/ml diacylglycerol. The mixture was incubated for 10 min at 37 °C, and the reaction was terminated by the addition of 600 µl of cold acetone. Bovine serum albumin (1 µg) was added, and after 30 min on ice, the precipitated protein was spun down, dissolved in sample buffer (125 mM Tris/HCl, pH 6.8, 5% (w/v) SDS, 12.5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol) and analyzed by SDS-PAGE (15% gel) followed by autoradiography.

**Regulation of the Phosphorylation of PI-TPα in Vitro**—For in vitro phosphorylation experiments, the 32P-labeled protein was reconstituted into the reaction volume of 60 µl containing 20 mM Tris/HCl, pH 7.5, 7.5 mM magnesium acetate, 10 µg/ml leupeptin, 10 µM ATP, and 1–2 µCi of [γ**−**32P]ATP. The Ca**2+**/phospholipid-independent phosphorylation was determined in the presence of 1 mM EGTA, and the Ca**2+**/phospholipid-dependent phosphorylation was determined in the presence of 1 mM Ca**2+**, 96 µg/ml phosphatidylserine, and 2.2 µg/ml diacylglycerol. The mixture was incubated for 10 min at 37 °C, and the reaction was terminated by the addition of 600 µl of cold acetone. Bovine serum albumin (1 µg) was added, and after 30 min on ice, the precipitated protein was spun down, dissolved in sample buffer (125 mM Tris/HCl, pH 6.8, 5% (w/v) SDS, 12.5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol) and analyzed by SDS-PAGE (15% gel) followed by autoradiography.

**Purification of PI-TPα—**The PI-TPα cDNA cloned into the pBluescript vector (pBlue-wtPI-TPα) (16) was used for site-directed mutagenesis using the Quickchange site-directed mutagenesis method according to the manufacturer’s instructions (Stratagene). Ser-166 was replaced by Ala using the following mutagenic oligonucleotides: sense primer, 5′-CCAGCAAATAATTTAAGGCT- GTCAAAAAACAGGACC-G-3′; antisense primer, 5′-GCGTCCTGTGTGTGAGCAGCCCTTAAATTTTCTGG-3′. The bold nucleotides encode the mutated amino acid (Ser-166 to Ala-166), and the underlined nucleotide is a mutation that does not result in a change in amino acid composition. A DraI restriction site. Incorporation of the mutagenic oligonucleotides into the construct (pBlue-wtPI-TPα(S166A)) was checked by restriction enzyme analysis and by sequencing. Both the mutated and wtPI-TPα cDNAs were cloned into the pET-15b expression vector. Expression of these constructs yielded wtPI-TPα or PI-TPα(S166A) fused to an N-terminal peptide containing six histidine residues. A mutant PI-TPα in which Ser-166 was replaced with Asp-166 was obtained in a similar way using the above oligonucleotides except that the bold nucleotides were replaced for GAT (sense primer) and ATC (antisense primer).
For phosphopeptide mapping the trichloroacetic acid pellet was dissolved in perchloric acid, and oxidation was performed for 1–2 h on ice. After lyophilization the sample was and incubated with TPCPK-trypsin in 50 mM ammonium bicarbonate (200 μg/ml) at 37 °C for 5 h. The incubation was repeated by the addition of fresh trypsin, and the sample was lyophilized. The phosphopeptides were separated on an anion-exchange TLC plates. In the first dimension electrophoresis was performed using the pH 1.9 buffer; in the second dimension TLC was performed in n-butanol:pyridine:glacial acetic acid:H2O (75:50:15:60 v/v/v/v). Radioactive phosphopeptides were identified by autoradiography.

Identification of the Phosphorylation Site—PI-TPα was phosphorylated as described above with the following changes. The ATP concentration was 1 mM with a trace of [γ-32P]ATP, and the incubation time was 2–4 h at 30 °C. The proteins were separated by SDS-PAGE, eluted, and precipitated with 10% trichloroacetic acid as described above. The pellet was digested with cyanogen bromide (2.5 mg/ml in 70% formic acid, 50 nmol cyanogen bromide/mmol protein) by incubation for 24 h in the dark at room temperature. After lyophilization, the sample was dissolved in 6 M guanidine HCl in 0.085% trifluoroacetic acid, and the peptides were separated on a reverse phase column C18/C18 (Amersham Pharmacia Biotech, SMART system) with a 0–60% (v/v) acetonitril. The radioactive peak was collected, and the N-terminal amino acid sequence of the 32P-labeled peptide was determined by automatic Edman degradation using the 476A protein sequencer (Applied Biosystems).

Preparation of Myc-tagged PI-TPα—The pBlue-wtPI-TPα, pBlue-PI-TPα(S166A) and pBlue-PI-TPα(S166D) constructs were used to generate Myc-tagged PI-TPα fusion proteins. The pBlue-PI-TPα constructs contained a SacI site upstream of the translational start codon, an NcoI restriction site around the translational stop codon, and a BamHI restriction site downstream of the translational stop codon. The SacI and NcoI sites were used to insert the linker encoding the Myc-tagged into the coding sequence. The linker consisted of two oligonucleotide primers carrying a SacI and an NcoI sticky end. The oligonucleotides used were: 5′-CATGGACAAAAACTTATTTCTGAGAAGATCTGC-3′ and 5′-CATGCGAGATCTTCTGAGAATAGTTCATGAC-3′. The underlined nucleotides of primer 2 represent the NcoI and an NcoI sticky end. The oligonucleotides were annealed at 60 °C for 15 min. After cooling to room temperature, the resulting linker was ligated into the pBlue-PI-TPα constructs that were previously digested with SacI and NcoI. The ensuing constructs were digested with SacI and BamHI, and the resulting DNA encoding Myc-tagged PI-TPα were ligated into the corresponding sites of the pBK-CMV expression vector. The obtained constructs were denoted as pBK-CMV-Myc-wtPI-TPα, pBK-CMV-Myc-PI-TPα(S166A), and pBK-CMV-Myc-PI-TPα(S166D). Expression of these constructs yields PI-TPα fused to an N-terminal peptide containing the 9E10 epitope (the peptide EQKLISEEDL) of the human c-Myc protein (Myc-tagged).

Transfection of NIH3T3 Cells with the Myc-tagged Constructs—NIH3T3 cells were seeded 24 h prior to transfection at 1 × 10^5 cells/cm^2. Cells were transfected with 2 μg of the pBK-CMV-Myc-PI-TPα constructs using the FuGENE6 Transfection Reagent kit according to the manufacturer’s instruction (Roche Molecular Biochemicals). The following day the cells were reseeded at approximately 5 × 10^4 cells/cm^2. After another 24 h, G418 (0.4 mg/ml) was added for selection of G418-resistant cells. Fresh medium containing G418 was added every 4 days, and resistant clones were identified after 3 weeks of growth.

**Immunolocalization**—The localization of endogenous PI-TPα, Myc-tagged wtPI-TPα, Myc-tagged PI-TPα(S166A), and Myc-tagged PI-TPα(S166D) in serum-starved (semi-quiescent) NIH3T3 cells was determined before and after stimulation with PMA or platelet derived growth factor (PDGF) as described in Ref. 15. Briefly, NIH3T3 cells were made semi-quiescent by replacing the growth medium with Dulbecco’s modified Eagle’s medium containing 0.5% newborn calf serum. After 2 days the cells were incubated for 15 min at 37 °C with PMA (50 ng/ml) or PDGF (20 ng/ml) and fixed with methanol (endogenous PI-TPα) or paraformaldehyde (Myc-tagged PI-TPα). Endogenous PI-TPα was visualized by indirect immunofluorescence using a polyclonal antibody directed against PI-TPα and goat-anti-rabbit-Cy3 as the second antibody. Myc-tagged PI-TPα was visualized using a mouse monoclonal antibody directed against the Myc-tagged and goat-anti-mouse tetramethyl rhodamine isothiocyanate as the second antibody.

**LysoPI Production in Vivo**—The effect of PMA and PDGF on the production of lysoPI in NIH3T3 cells before and after stimulation with PMA or PDGF was determined as described in Ref. 12. Briefly, NIH3T3 cells were cultured in a 6-well plate to 90% confluency. The cell cultures were incubated for 48 h with 2 μCi of [3H]-myo-inositol in HEPES-buffered DF medium without inositol containing 2% dialyzed newborn calf serum. Cultures were washed twice with phosphate-buffered saline and incubated for 10 min at 37 °C with DF medium (without inositol) containing 0.3% bovine serum albumin and 10 mM LiCl. Subsequently, PMA (50 ng/ml) or PDGF (20 ng/ml) was added, and the incubation was continued for another 15 min. The cells were washed twice with phosphate-buffered saline and scraped in 1 ml of 20 °C methanol. The [3H]inositol phospholipids were extracted and analyzed as described previously (23).

**RESULTS**

Kinetic Analysis of Phosphorylation—The PKC-dependent phosphorylation of the charge isomers PI-TPαI and II from bovine brain was determined in vitro as a function of concentration. From the Lineweaver-Burk plot it was calculated that the V_max values about 1 order of magnitude higher V_max values about 1 order of magnitude higher than PI-TPαII. (Fig. 1A). The K_m of either reaction was comparable (0.65 and 0.72 μM, respectively). This implies that the affinity of rat brain PKC for both isomers is the same, yet PI-TPα containing a PC molecule is phosphorylated at a faster rate than the protein containing a PI molecule. To confirm these results we have done similar experiments on mouse recPI-TPα. RecPI-TPα contains a PG molecule that can be readily exchanged for either PI or PC (16). Phosphorylation of the charge isomers of recPI-TPα by PKC confirmed that the PC-containing protein was phosphorylated at a faster rate than the PI-containing protein (Fig. 1B). In addition, phosphorylation of the PG-containing protein could be compared with that of the PC-containing protein (data not shown). In comparison with the bovine PI-TPα the mouse recPI-TPα was more resistant to phosphatase, with V_max values about 1 order of magnitude higher and K_m values 1 order of magnitude lower (0.1 μM).

Phosphopeptide Mapping and Phosphoamino Acid Analysis—Two-dimensional analysis of tryptic 32P-labeled peptides showed that PI-TPαI, PI-TPαII and recPI-TPα have one major...
phosphopeptide in common as well as a minor one. The phosphopeptide map of PI-TPαII showed three additional minor spots (Fig. 2). Phosphoamino acid analysis of the in vitro phosphorylated proteins demonstrated that all three PI-TPαs are mainly phosphorylated on serine (Fig. 3). The immunoprecipitated PI-TPα from PMA-stimulated 32P-labeled Swiss mouse 3T3 cells was purified by SDS-PAGE. Phosphopeptide mapping and phosphoamino acid analysis of 32P-labeled PI-TPα showed that in vivo PI-TPα was exclusively phosphorylated on serine (data not shown). In line with this result, the phosphopeptide map is very similar to that of recPI-TPα (data not shown). Figure 4 shows that Ser-166 in PI-TPα was replaced with Ala. The resulting His6-tagged PI-TPα(S166A) protein was cleaved by cyanogen bromide, and the phosphopeptides were separated on a reversed phase column. The 32P label was detected in one peak that, as shown by automated Edman degradation, represented the cyanogen bromide peptide only Ser-166 is the predicted PKC amino acid residue of phosphorylation and that in the labeled phosphoamino acid analysis showed serine as the preferred amino acid residue of phosphorylation and that in the labeled phosphoamino acid analysis showed serine as the preferred amino acid residue of phosphorylation and that in the labeled phosphoamino acid analysis showed serine as the preferred amino acid residue of phosphorylation and that in the labeled phosphoamino acid analysis showed serine as the preferred amino acid residue of phosphorylation and that in the labeled phosphoamino acid analysis showed serine as the preferred

indicates that Ser-166 is important for transfer activity. To investigate the possibility that the lack of transfer activity was due to incorrect folding during expression in E. coli, we have carried out refolding experiments on inclusion bodies according to standard procedures. In agreement with the above observations, Hisα-tagged PI-TPα(S166A) could not be activated, whereas refolded Hisα-tagged PI-TPα(S166A) was active.

Under optimal conditions 10–15% of PI-TPα was found to be phosphorylated by PKC in vitro (15). In agreement with this relatively low level of phosphorylation, the phospholipid transfer activity was not affected by PKC treatment (data not shown). To further examine the effect of phosphorylation on transfer activity, a mutant of PI-TPα was made in which Ser-166 was replaced with Asp to mimic phosphorylated serine. As shown in Fig. 5, PI-TPα(S166D) was not active in the phospho-

Fig. 2. Phosphopeptide maps of bovine PI-TPα and II, mouse recPI-TPα phosphorylated in vitro, and mouse PI-TPα phosphorylated in vivo. 32P-Labeled PI-TPα was degraded by trypsin, and the phosphopeptides were separated on a thin layer plate followed by autoradiography as described under “Experimental Procedures.” The arrow indicates the origin.

Fig. 3. Phosphoamino acid analysis of PI-TPα, PI-TPαII, and recPI-TPα. 32P-Labeled PI-TPα was hydrolyzed by 6 M HCl and subjected to two-dimensional separation on a thin layer plate. The positions of the origin (as indicated by the arrow), phosphoserine (S), phosphothreonine (T), phosphotyrosine (Y), and inorganic phosphate (P) are indicated.

Fig. 4. Phosphorylation of wtPI-TPα and PI-TPα(S166A). PI-TPα was phosphorylated as described under “Experimental Procedures” and subjected to SDS-PAGE analysis (A) and autoradiography (B). Lanes 1 and 2, 1 μg of PI-TPα(S166A); lanes 3 and 4, 2 μg of PI-TPα(S166A); lanes 5 and 6, 1 μg of wtPI-TPα; lanes 7 and 8, 2 μg of wtPI-TPα; lanes 9 and 10, PKC control. The samples in the odd-numbered lanes were incubated in the absence of Ca2++, phosphatidylinositol, and diaoylglycerol, and those in the even-numbered lanes were incubated in the presence of Ca2++, phosphatidylinositol, and diaoylglycerol.

Fig. 5. In vitro phospholipid transfer activity of mutant and wtPI-TPα. The phospholipid transfer activity of the PI-TPα was assayed as described under “Experimental Procedures” by measuring the transfer of fluorescently labeled PI (Pyr-PI) from quenched donor vesicles to acceptor vesicles. The donor vesicles in the cuvette were subsequently added the acceptor vesicles (arrow I), bovine serum albumin (arrow II), and different PI-TPαs (arrow III): Line 1, wtPI-TPα; line 2, PI-TPα(S152A); line 3, PI-TPα(S166A); Line 4, PI-TPα(S166D).
lipid transfer activity assay, suggesting that the phosphorylation of Ser-166 may constitute a regulatory step.

As shown in Ref. 12, wtPI-TPα stimulates lysoPI formation in an *in vitro* assay. Under comparable conditions PI-TPα(S166A) had no effect (data not shown). From this we conclude that PI-transfer activity is a prerequisite for PI-TPα to stimulate lysoPI formation.

**Relocalization and LysoPI Formation—** Stimulation of NIH3T3 cells with PMA for 15 min resulted in an extensive relocalization of PI-TPα to perinuclear membrane structures (Fig. 6). The same relocalization was observed after stimulation with PDGF (data not shown). This is in agreement with a previous study on the relocalization of PI-TPα in Swiss mouse 3T3 cells (15). In the latter study it was shown that these perinuclear structures were Golgi-like. To determine whether phosphorylation of PI-TPα was required for the relocalization to the Golgi complex, NIH3T3 cells were transfected with the pBK-CMV-Myc-wtPI-TPα, PI-TPα(S166A), and PI-TPα(S166D) constructs. The localization of the Myc-tagged PI-TPαs was determined in serum-starved cells using an antibody against the Myc-tagged. Similar to the endogenous PI-TPα, Myc-tagged wtPI-TPα was localized throughout the cytosol and the nucleus and relocated to perinuclear Golgi structures upon stimulation by PMA (data not shown). Myc-tagged PI-TPα(S166A) and Myc-tagged PI-TPα(S166D) were also localized in the cytosol and the nucleus. However, these mutants did not relocalize upon stimulation by PMA, suggesting that phosphorylation of Ser-166 is required for the relocalization to the Golgi. Given that the expression of PI-TPα is involved in the regulation of phospholipase A-dependent formation of lysoPI (12), we have determined the cellular amount of lysoPI under the above conditions of PI-TPα translocation. Stimulation by PMA and PDGF increased the amount of lysoPI 2- and 3-fold, respectively (Fig. 7). No significant effects were observed on the levels of PI 4-phosphate and PI 4,5-bisphosphate.

**DISCUSSION**

Analysis of the amino acid sequence of mammalian PI-TPα showed the presence of five possible sequence motifs that are consensus phosphorylation sites for PKC (i.e. Thr-59, Thr-169, Thr-198, Thr-251, and Ser-166) (15). A review on specific phosphorylation sites for various PKC isoforms indicated that Ser-166 is the only putative site (24). In this study we have proven by peptide analysis and site-directed mutagenesis that indeed the phosphorylation of PI-TPα by rat brain PKC *in vitro* was restricted to Ser-166. Stimulation of PKC in Swiss mouse 3T3 cells by phorbol ester (PMA) also phosphorylated a serine residue of PI-TPα. Given that the tryptic peptide maps of the *in vitro* and *in vivo* phosphorylated PI-TPαs were comparable, we presume that Ser-166 is the site of PKC-dependent phosphorylation in intact cells.

Replacement of Ser-166 with an Ala or Asp residue yielded a PI-TPα species, which *in vitro* completely lacked PI and PC transfer activity. For comparison, replacement of Ser-152 with an Ala residue had no effect on the transfer activity. This indicates that the activity of PI-TPα is dependent on the presence of Ser-166. This residue may be essential for a correct folding of the active protein during its expression in *E. coli.* It is possible that concomitant with the expression, Ser-166 may form a hydrogen bond with the polar head group of the PG molecule that is normally present in recombinant wtPI-TPα (16). In the case of Ala-166 or Asp-166, this hydrogen bond cannot be formed, resulting in a PI-TPα that cannot interact with PG. This interaction may be important for PI-TPα to fold properly during its synthesis in *E. coli.* Reactivation of native PI-TPα expressed in *E. coli* from inclusion bodies was only possible when phospholipid was present in the refolding buffer. Under these conditions of refolding, inclusion bodies of PI-TPα(S166A) did not yield an active protein. In addition, comparison of holo- and apo-species (with or without a phospholipid ligand, respectively) of native PI-TPα indicated that binding of a phospholipid ligand is required for obtaining the proper, more compact structure of holo-PI-TPα. Apo-PI-TPα demonstrated a significant relaxation of the tertiary structure (25). In a previous study it was reported that mutation of the putative PKC phosphorylation site Thr-59 abolished the PI but not the PC transfer activity (26). Based on this observation a model was proposed where the phosphorylation/dephosphorylation of Thr-59 was presented as a key event in the regulation of phospholipid transfer activity *in situ.* However, because we have not found any evidence for the phosphorylation of a threonine residue, it remains to be established whether Thr-59 plays any role in this process.

Apart from being essential for proper folding and, hence, for *in vitro* phospholipid transfer activity, Ser-166 is also the single site of PKC phosphorylation. Very interestingly, the rate of phosphorylation was dependent on the phospholipid ligand bound to PI-TPα. Both for bovine and mouse PI-TPαs, it was observed that the *V*max was 2–3-fold higher with PC than with PI as ligand. Because the *Km* values were comparable, we concluded that the affinity of PKC for PI-TPα was not affected by the ligand, yet that the charge difference between PI and PC resulted in a different rate of phosphorylation. It is as yet impossible to determine the phosphorylation of PI-TPαs and II *in situ* as conventional methods fail to distinguish between these charge isomers. From the three-dimensional structure of the yeast PI-TP analogue (SEC14p), we know that the polar head group of the bound phospholipid molecule is exposed at the surface of the protein (27). If we assume that this is also the case...
Regulation of the Phosphorylation of PI-TPα

This model presents an explanation for the relationship between the PKC-dependent phosphorylation of PI-TPα and the formation of PI-binding vesicles in reconstituted systems (2, 28–30). It has been proposed that PKC-dependent phosphorylation of PI-TPα results in the formation of PI-containing vesicles through the local conversion of PI into metabolites that cause the remodeling of the cytoskeleton. In crystallization studies of PI-TPα, we observed that the conditions at which crystals were obtained were dependent on the bound phospholipid, suggesting a direct effect on the surface charge and conformation. 1 In the present study, the recombinant mouse PI-TPα was a better substrate for the rat brain PKC compared with the bovine PI-TPα. Apparently, the minor differences in isoelectric point and primary structure (16) have an effect on the in vitro phosphorylation by rat brain PKC.

In a previous study, we have shown that activation of PKC in Swiss mouse 3T3 cells induces a translocation of PI-TPα from the cytosol to the Golgi complex (15). A similar translocation to the Golgi was observed when NIH3T3 cells were activated by PMA or PDGF (Fig. 6). We presume that phosphorylation of PI-TPα is required to make PI-TPα interact with the Golgi. This raises the question of what function PI-TPα may fulfill at the Golgi. In previous studies PI-TPα was shown to play an essential role in the ATP-dependent formation of transport vesicles from the trans–Golgi network in reconstituted systems (2, 28–30). It has been proposed that the formation of these vesicles involves the local conversion of PI into metabolites that cause the remodelling of the phospholipid bilayer in the vicinity of a coated bud (28). PI-TPα would play a role in this conversion by delivering PI as a substrate to PI kinases (31). However, whether PI-TPα fulfills a role in the phosphorylation of PI in intact cells has not been confirmed. Studies with NIH3T3 cells overexpressing PI-TPα have indicated that the intracellular levels of lysoPI and further metabolites glycerophosphoinositol and its further metabolites have been shown to act as a mitogen in certain cell lines (32) and may possibly contribute to the enhanced growth rate of these PI-TPα overexpressers (12). In cells overexpressing PI-TPα, effects on PI metabolism were not observed. Concomitant with the translocation of PI-TPα to the Golgi, activation of wtNIH3T3 cells by PDGF and PMA resulted in a 2–3-fold increase of lysoPI (Fig. 7). In view of our evidence that levels of lysoPI are controlled by the amount of PI-TPα expressed, we assume that the PKC-dependent translocation of PI-TPα to the Golgi results in more PI becoming available for degradation by the phospholipase. Based on this hypothesis, we propose a model explaining how the phosphorylation of PI-TPα results in an enhanced lysoPI formation. As shown in the model presented in Fig. 8, the receptor-controlled activation of PKC leads to a more rapid phosphorylation of the PC-containing PI-TPα than of the PI-containing PI-TPα. The phosphorylated form of PI-TPα II is then translocated to the Golgi where in view of the high preference for PI, the protein unloads its PC molecule and binds a PI molecule. It can be expected that upon interaction with the Golgi, the phospholipid exchange reaction and dephosphorylation of PI-TPα by a protein phosphatase occurs. A similar protein phosphatase was proposed in the model by Alb et al. (26). After its release from the Golgi, PI-TPα delivers its bound PI to the PI-specific phospholipase A2 to be degraded. The effect of the phosphorylation/dephosphorylation of PI-TPα is that upon receptor activation at the plasma membrane there is a rapid and controlled increase of PI available for metabolism. The important implication of this model is that the relative amount of PI and PC-containing PI-TPα as controlled by the accessible PI and PC pools in the cell can be shifted toward the PI-containing PI-TPα as a result of PKC activation.

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REFERENCES
1. Fonesma, A., Cunningham, E., Prosser, S., Tan, S. K., Swigart, P., Thomas, G., Hsuan, J., and Cockcroft, S. (1996) Curr. Biol. 6, 730–738
2. Ohashi, M., de Vries, K. J., Frank, B., Snoek, G. T., Bankaitis, V. A., Wirtz, K. W. A., and Hutten, W. B. (1995) Nature 377, 544–547
3. Thomas, G. M., Cunningham, E., Fonesma, A., Ball, A., Toty, N. F., Truong, O., Hsuan, J. J., and Cockcroft, S. (1995) Cell 74, 919–928
4. Cockcroft, S., Ball, A., Fonesma, A., Hara, S., Jones, D., Prosser, S., and Swigart, P. (1997) Biochem. Soc. Trans. 25, 1125–1131
5. Panaretou, B., Domin, J., Cockcroft, S., and Waterfield, M. D. (1997) J. Biol. Chem. 272, 2477–2485
6. Kular, G., Loubtchenkov, M., Swigart, P., Whatmore, J., Ball, A., Cockcroft, S., and Wetzaker, R. (1997) Biochem. J. 325, 299–301
7. de Vries, K. J., Heinrichs, K. A., Cunningham, E., Brunink, F., Westerman, J., Somerharju, P., Cockcroft, S., Wirtz, K. W. A., and Snoek, G. T. (1995) Biochem. J. 310, 643–649
8. de Vries, K. J., Westerman, J., Bastiaensen, P. J., Jovin, T. M., Wirtz, K. W. A., and Snoek, G. T. (1996) Exp. Cell. Res. 227, 33–39
9. Tanaka, S., and Hosaka, K. (1994) J. Biochem. (Tokyo) 115, 981–984
10. Wirtz, K. W. A. (1995) Biochem. J. 323, 353–360
11. Wirtz, K. W. A., and Snoek, G. T. (1995) J. Biol. Chem. 270, 14263–14266
12. Snoek, G. T., Berrie, C. P., Geijtenbeek, T. B., van der Helm, H. A., Cadee, J. A., Iurisci, C., Corda, D., and Wirtz, K. W. A. (1999) J. Biol. Chem. 274, 35393–35399
13. Van Tiel, C. M., Luberto, C., Snoek, G. T., Hannun, Y. A., Wirtz, K. W. A. (2000) Biochem. J. 346, 537–543
14. Voziyan, P. A., Tremblay, J. M., Yarbrough, L. R., and Helmkamp, G. M., Jr. (1993) Anal. Biochem. 215, 643–656
15. Alb, J. G. J., Gedvilaite, A., Carter, R. T., Skinner, H. B., and Bankaitis, V. A.
Regulation of the Phosphorylation of PI-TPα

27. Sha, B., Phillips, S. E., Bankaitis, V. A., and Luo, M. (1998) Nature 391, 506–510
28. Simon, J. P., Morimoto, T., Bankaitis, V. A., Gottlieb, T. A., Ivanov, I. E., Adesnik, M., and Sabatini, D. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11181–11186
29. Paul, K. S., Bogan, A. A., and Waters, M. G. (1998) FEBS Lett. 431, 91–96
30. Jones, S. M., Alb, J. G., J., Phillips, S. E., Bankaitis, V. A., and Howell, K. E. (1998) J. Biol. Chem. 273, 10349–10354
31. Cockcroft, S. (1997) FEBS Lett. 410, 44–48
32. Falasca, M., Marino, M., Carvelli, A., Iurisci, C., Leoni, S., and Corda, D. (1996) Eur. J. Biochem. 241, 386–392
