THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 25, Issue of June 21, pp. 22447–22452, 2002

Printed in U.S.A.

The Golgi Localization of Phosphatidylinositol Transfer Protein β Requires the Protein Kinase C-dependent Phosphorylation of Serine 262 and Is Essential for Maintaining Plasma Membrane Sphingomyelin Levels*

Received for publication, February 14, 2002 and in revised form, April 8, 2002
Published, JBC Papers in Press, April 12, 2002, DOI 10.1074/jbc.M201532200

Claudia M. van Tiel‡, Jan Westerman, Marten A. Paasman, Martha M. Hoebens, Karel W. A. Wirtz, and Gerry T. Snoek

From the Center for Biomembranes and Lipid Enzymology, Department of Lipid Biochemistry, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Recombinant mouse phosphatidylinositol transfer protein (PI-TP)β is a substrate for protein kinase C (PKC)-dependent phosphorylation *in vitro*. Based on site-directed mutagenesis and two-dimensional tryptic peptide mapping, Ser^{262} was identified as the major site of phosphorylation and Ser^{165} as a minor phosphorylation site. The phospholipid transfer activities of wild-type PI-TPβ and PI-TPβ(S262A) were identical, whereas PI-TPβ(S165A) was completely inactive. PKC-dependent phosphorylation of Ser^{262} also had no effect on the transfer activity of PI-TPβ. To investigate the role of Ser^{262} in the functioning of PI-TPβ, wtPI-TPβ and PI-TPβ(S262A) were overexpressed in NIH3T3 fibroblast cells. Two-dimensional PAGE analysis of cell lysates was used to separate PI-TPβ from its phosphorylated form. After Western blotting, wtPI-TPβ was found to be 85% phosphorylated, whereas PI-TPβ(S262A) was not phosphorylated. In the presence of the PKC inhibitor GF 109203X, the phosphorylated form of wtPI-TPβ was strongly reduced. Immunolocalization showed that wtPI-TPβ was predominantly associated with the Golgi membranes. In the presence of the PKC inhibitor, wtPI-TPβ was distributed throughout the cell similar to what was observed for PI-TPβ(S262A). In contrast to wtPI-TPβ overexpressors, cells overexpressing PI-TPβ(S262A) were unable to rapidly replenish sphingomyelin in the plasma membrane upon degradation by sphingomyelinase. This implies that PKC-dependent association with the Golgi complex is a prerequisite for PI-TPβ to express its effect on sphingomyelin metabolism.

Eukaryotic phosphatidylinositol transfer proteins (PI-TPs)[1] belong to a family of highly conserved proteins that are able to transfer phospholipids between membranes or from membrane to enzyme (1). In mammalian tissues at least two isoforms, PI-TPα and PI-TPβ, are found. PI-TPα is able to transfer phosphatidylinositol (PI) and, to a lesser extent, phosphatidylcholine (PC) (2–6) and is mainly localized in the cytosol and in the nucleus (7). Similar to PI-TPα, PI-TPβ is able to transfer PI and PC but is also able to transfer sphingomyelin (SM) (8). PI-TPβ is mainly associated with the Golgi apparatus (7). The primary sequences of PI-TPα and PI-TPβ are very similar, with an identity of 77% and a similarity of 94% (9).

To date, little is known about the exact cellular function of PI-TPα and PI-TPβ. In a cell-free system containing *trans*-Golgi membranes, both PI-TPα and PI-TPβ stimulated the formation of constitutive secretory vesicles and immature granules (10). In permeabilized, cytosol-depleted HL60 cells, both isoforms restored GTPγS-stimulated protein secretion and phospholipase C-mediated inositol lipid signaling (11, 12). In these assays, PI-TPα and PI-TPβ were found to function equally well despite their different biochemical properties and cellular localizations. On the other hand, NIH3T3 cells with increased expression of either PI-TPα or PI-TPβ demonstrated remarkable differences in lipid metabolic pathways. Cells overexpressing PI-TPα (SPIα cells) showed an enhanced PLA_2-mediated PI degradation resulting in increased levels of lyso-PI, glycerophosphoinositol, Ins(1)P, and Ins(2)P (13). This was not observed in cells overexpressing PI-TPβ (SPIβ cells). However, in SPIβ cells (but not in SPIα cells) it was shown that under conditions in which plasma membrane SM was hydrolyzed to ceramide by exogenous sphingomyelinase, PI-TPβ was involved in maintaining the steady-state levels of SM (14). It was recently postulated that PI-TPβ plays a key role in SM metabolism, making it a potential regulator of pathways for diacylglycerol production and consumption in the mammalian *trans*-Golgi network (15). Disruption of the PI-TPβ gene in mice leads to early failure in embryonic development (16).

In search of mechanisms by which PI-TP activity is regulated, PI-TPα was shown to be phosphorylated by protein kinase C *in vitro* as well as *in vivo* (17, 18). PI-TPα was exclusively phosphorylated on Ser^{166}, with the PC-carrying form of PI-TPα more readily phosphorylated than the PI-carrying form (18). Furthermore, in NIH3T3 cells, PI-TPα was translocated from the cytosol to the Golgi membranes upon phosphorylation after stimulation with phorbol ester. This relocalization of PI-TPα coincided with an increased level of intracellular lyso-PI, indicating the activation of a PI-specific PLA_2 (17, 18). Based on these findings, a model was proposed for the regulation of PI-TPα by PKC-dependent phosphorylation. In contrast to PI-TPα, PI-TPβ purified from bovine or rat brain could not be

* This work was supported by The Netherlands Organization for Scientific Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 31-30-2533952; Fax: 31-30-2533151; E-mail: c.vantiel@chem.uu.nl.

[1] The abbreviations used are: PI-TP, phosphatidylinositol transfer protein; wtPI-TP, wild-type PI-TP; PI, phosphatidylinositol; PC, phosphatidylcholine; Ins, inositol; SM, sphingomyelin; PKC, protein kinase C; PA, phosphatidic acid; PS, phosphatidylserine; TNP-PE, trinitrophenyl phosphatidylethanolamine; GAR-PO, goat anti-rabbit peroxidase; bSMase, bacterial sphingomyelinase; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PLA_2, phospholipase A_2; Pyr, pyrene; GTPγS, guanosine 5′-*O*-(thiotriphosphate); TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

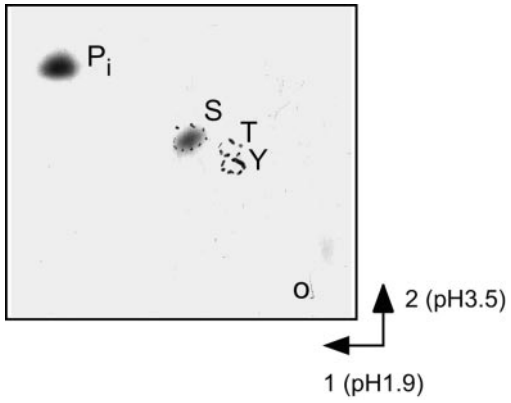

FIG. 1. **Phosphoamino acid analysis of His_6-tagged PI-TPβ.** ^{32}P-labeled His_6-tagged PI-TPβ was hydrolyzed by HCl and subjected to two-dimensional separation on a thin-layer plate. The phosphoamino acid standard spots were visualized with ninhydrin. The positions of the origin (*o*), phosphoserine (*S*), phosphothreonine (*T*), phosphotyrosine (*Y*), and inorganic phosphate (P_i) are indicated.

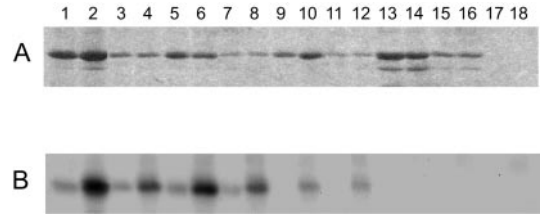

FIG. 2. **PKC-dependent phosphorylation of wild-type and mutant His_6-tagged PI-TPβ.** PI-TPβ (1 and 2 μg) was phosphorylated by PKC and subjected to SDS-PAGE analysis (*panel A*) and autoradiography (*panel B*). *Lanes 1–4*, wtPI-TPβ; *lanes 5–8*, PI-TPβ(S165A); *lanes 9–12*, PI-TPβ(S262A); *lanes 13–16*, PI-TPβ(S165A/S262A); *lanes 17–18*, PKC control. The samples in the *odd-numbered lanes* were phosphorylated in the absence of Ca^{2+} and PS. The samples in the *even-numbered lanes* were phosphorylated in the presence of Ca^{2+} and PS.

phosphorylated despite the fact that it contains the same serine residue (Ser^{165}) and an additional putative PKC phosphorylation site (Ser^{262}) not present in PI-TPα (8, 17, 19).

In this study, we report that murine PI-TPβ can be phosphorylated by PKC *in vitro* as well as *in situ*. The major site of phosphorylation was Ser^{262}, whereas Ser^{165} was a minor site. By site-directed mutagenesis we have shown that Ser^{165} is essential for the lipid transfer activity of the protein, whereas phosphorylation of Ser^{262} is required for the association of PI-TPβ with the Golgi membranes. This latter residue is also essential for the ability of PI-TPβ to maintain steady-state levels of SM in the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—PI, phosphatidic acid (PA), phosphatidylserine (PS), egg yolk PC, trinitrophenyl phosphatidylethanolamine (TNP-PE), ATP, goat anti-rabbit IgG conjugated with peroxidase (GAR-PO), bacterial sphingomyelinase (bSMase, from *Staphylococcus aureus*), GF 109203X, phosphoserine, phosphothreonine, and phosphotyrosine were obtained from Sigma. The pBluescript SK^- vector, the pBK-CMV vector, and the QuikChange site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA). The oligonucleotides were synthesized by Eurogentec, Belgium. The pET15b vector was obtained from Novagen, Madison, WI. The pEYFP-C1 vector was from CLONTECH, Palo Alto, CA. The *Escherichia coli* strain BL21(DE3) was obtained from Dr. J. H. Veerkamp (Dept. of Biochemistry, University of Nijmegen, The Netherlands). Ni^{2+}-Hybond matrix was from Invitrogen. [γ-^{32}P]ATP (3000 Ci/mmol) and dNTPs were obtained from Amersham Biosciences. Cellulose TLC plates and TPCK-trypsin were purchased from Merck. Bio-Lyte carrier ampholytes were from Bio-Rad. *N*-pyrenyl-tetradecanoyl-SM (Pyr-SM), 1-palmitoyl-2-pyrenyl-decanoyl-PI (Pyr-PI), and 1-palmitoyl-2-pyrenyl-decanoyl PC (Pyr-PC) were a kind gift from Dr. P. Somerharju (University of Helsinki, Finland). GARCy3 was obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.

Site-directed Mutagenesis of PI-TPβ—The PI-TPβ cDNA cloned into the pBluescript SK^- vector (14) was used for site-directed mutagenesis using the QuikChange site-directed mutagenesis method according to the manufacturer's instruction. Ser^{165} was replaced by Ala using the following mutagenic oligonucleotides: sense 165 primer, 5′-CCTGCATTATTCCAC**GCA**GTGAAGACCAAGAGA-3′ and antisense 165 primer, 5′-TCTCTTGGTCTTCAC**TGC**GTGGAATAATGCAGG-3′. The bold nucleotides encode the mutated amino acid (Ser^{165} to Ala^{165}) The underlined nucleotides are mutations that do not result in a change in amino acid composition, but they introduce a *Dra*III restriction site. The mutated construct is denoted as pBluePI-TPβ(S165A).

Ser^{262} was replaced by Ala using the following mutagenic oligonucleotides: sense 262 primer, 5′-ATGCGTAAGAAGGGT**GCG**GTCCGAGGCACGTCG-3′ and antisense 262 primer, 5′-CGACGTGCCTCGGAC**CGC**ACCCTTCTTACGCAT-3′. The bold nucleotides encode the mutated amino acid (Ser^{262} to Ala^{262}). This mutation also results in the introduction of an *Rsr*II restriction site. The resulting construct is denoted as pBluePI-TPβ(S262A). A mutant in which both Ser^{165} and Ser^{262} were replaced by Ala was generated using the same primers as for the mutation of Ser^{262} to Ala^{262} with pBluePI-TPβ(S165A) as target DNA in the mutagenesis reaction. The resulting construct is denoted as pBluePI-TPβ(S165A/S262A). Incorporation of the mutagenic oligonucleotides into the construct was checked by restriction enzyme analysis and by DNA sequencing. The three mutated and the wtPI-TPβ cDNAs were cloned into the pET15b expression vector. Expression of these pET15b-PI-TPβ constructs yielded wtPI-TPβ or mutant PI-TPβ fused to an N-terminal peptide containing six histidine residues (His_6 tag).

Purification of Wild-type and Mutant His_6-tagged PI-TPβ—The *E. coli* strain BL21(DE3) was transformed by the pET15b-PI-TPβ constructs. A 50-ml culture, grown overnight in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin was used to inoculate 2 liters of LB medium (also containing 50 μg/ml ampicillin). The culture was grown at 18 °C for 24 h, and the His_6-tagged PI-TPβs (wt and mutant proteins) were purified from these by Ni^{2+}-Hybond affinity chromatography. After chromatography, the fractions were assayed for PI transfer activity and immunoreactivity (enzyme-linked immunosorbent assay). After the final purification step, the fractions containing His_6-tagged PI-TPβ were combined and concentrated to 10 ml. The purified His_6-tagged PI-TPβs were stored in 52% (v/v) glycerol at −20 °C.

Phospholipid Transfer Activity Assay—PI, PC, and SM transfer activities were determined in a continuous fluorescence assay using donor vesicles consisting of either Pyr-PI, Pyr-PC or Pyr-SM and PA, egg-PC, and TNP-PE (10:10:70:10, mol%) and acceptor vesicles consisting of PC and PA (95:5, mol%) (3, 20). Measurements were performed using a fluorimeter (Photon Technology International) equipped with a thermostated cuvette holder and a stirring device.

Purification of Protein Kinase C—PKC was purified from rat brain by a modified procedure previously described by Huang *et al.* (21). 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 III as substrate. The purified enzyme is stable for several months when kept at −80 °C in 50% glycerol and 0.01% Triton X-100.

Phosphorylation of PI-TPβ in Vitro by Protein Kinase C—His_6-tagged PI-TPβ (1 and 2 μg) was phosphorylated in a 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-^{32}P]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 3.2 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.

In some experiments, phosphorylation of PI-TPβ (2 μg/assay) was optimized by increasing the amount of ATP to 1 mM and by extending the time of incubation to 30 min. To estimate the extent of phosphorylation, the samples were submitted to SDS-PAGE. The band containing ^{32}P-labeled PI-TPβ was cut out from the dried gel, and the labeled protein was eluted by incubation with Soluene-350 (Packard Bioscience) for 2 h at 50 °C. Radioactivity was determined by liquid scintillation counting. The stoichiometry of phosphorylation was calculated from the PI-TPβ ^{32}P radioactivity, the amount of protein applied to the gel, and the specific activity of ATP.

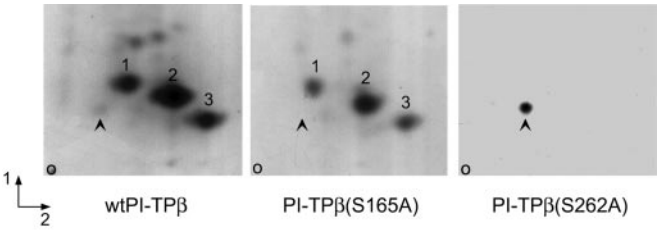

FIG. 3. **Phosphopeptide maps of His_6-tagged wtPI-TPβ, His_6-tagged PI-TPβ(S165A), and His_6-tagged PI-TPβ(S262A).** Phosphorylated PI-TPβ was degraded by trypsin, and the phosphopeptides were separated on a thin-layer plate followed by autoradiography. The position of the origin (*o*) is shown. The *arrowheads* indicate the position of the phosphopeptide containing Ser^{165}. *Spots 1–3* indicate the phosphopeptides containing Ser^{262}.

Phosphopeptide and Phosphoamino Acid Analysis—After identification by autoradiography, the ^{32}P-labeled bands were excised from the dried gel and eluted as described by Boyle *et al.* (22). Briefly, the gel slices were homogenized in 50 mM ammonium bicarbonate, pH 7.3–7.6. SDS (final concentration, 0.1%) and 2-mercaptoethanol (final concentration, 1%) were added, and the sample was boiled for 5 min. After incubation of the mixture at 37 °C for 2 h the gel was spun down, and the supernatant containing the ^{32}P-labeled proteins was collected. A second elution with 0.1% SDS and 1% 2-mercaptoethanol was carried out on the gel pellet. Carrier protein (boiled RNase, 10 μg) and trichloroacetic acid (final concentration, 10%) were added to the combined supernatant fractions, and the samples were incubated on ice for 1 h. The trichloroacetic acid precipitate was washed with cold ethanol and dried. For phosphoamino acid analysis, the pellet was dissolved in 6 M HCl and hydrolyzed for 1 h at 110 °C. The HCl was removed by lyophilization, and the pellet was dissolved in pH 1.9 buffer: glacial acetic acid/formic acid/H_2O (88%) (78:25:897, v/v/v). A mixture of phosphoserine, phosphothreonine, and phosphotyrosine (1 μg of each) was added. The ^{32}P-labeled phosphoamino acids were separated by two-dimensional electrophoresis on 20 × 20-cm cellulose TLC plates. The first dimension was in buffer, pH 1.9, and the second dimension was in glacial acetic acid/pyridine/H_2O (50:5:945, v/v/v), pH 3.5. After electrophoresis the plates were dried, the phosphoamino acids were visualized by staining with 0.2% (w/v) ninhydrin in acetone, and the ^{32}P-labeled amino acids were identified by autoradiography.

For phosphopeptide mapping, the trichloroacetic acid pellet was dissolved in performic acid, and oxidation was performed for 1–2 h on ice. After lyophilization the sample was incubated with TPCK-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 cellulose TLC plates. In the first dimension, electrophoresis was performed using the pH 1.9 buffer; in the second dimension, TLC was performed in *n*-butyl alcohol/pyridine/glacial acetic acid/H_2O (75:50:15:60, v/v/v/v). Radioactive phosphopeptides were identified by autoradiography.

pBK-CMV-PI-TPβ Constructs for Transfection of NIH3T3 Fibroblast Cells—PI-TPβ (S262A) cDNA was isolated from the pBluescript SK^- vector by digestion with *Bam*HI and *Sac*I and cloned into the corresponding sites of the pBK-CMV expression vector. PI-TPβ expression was regulated by the cytomegalovirus immediate early promotor, and the SV40 poly(A) adenylation signal provided the signal for termination of eukaryotic transcription and polyadenylation.

Cell Culture and Transfection—NIH3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and buffered with $NaHCO_3$ (44 mM) at 5% CO_2 in a humidified atmosphere. NIH3T3 cells were transfected using the method of Chen and Okayama (23). Briefly, cells were seeded 5 h prior to transfection at 1×10^4 cells/cm^2 and then transfected with 20 μg of the pBK-CMV-PI-TPβ constructs. 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, denoted as SPIβ(S262A) cells, were identified after 3 weeks of growth.

Gel Electrophoresis and Western Blotting—The PI-TPβ content of several G418-resistant clones was analyzed by immunoblotting with anti-PI-TPβ antibodies. Cells were washed twice with PBS and lysed in 500 μl of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% (w/v) Nonidet P-40). After centrifugation at 17,500 × *g* for 10 min at 4 °C, the protein concentration of the supernatant was determined using the Lowry assay (24). A 150-μg portion of protein was subjected to SDS-PAGE on a 15% gel and analyzed by Western blotting using a PI-TPβ-specific antibody. The PI-TPβ levels on the immunoblot were quantified using a Bio-Rad GS700 imaging densitometer equipped with an integrating program. Known amounts of PI-TPβ were used as a standard.

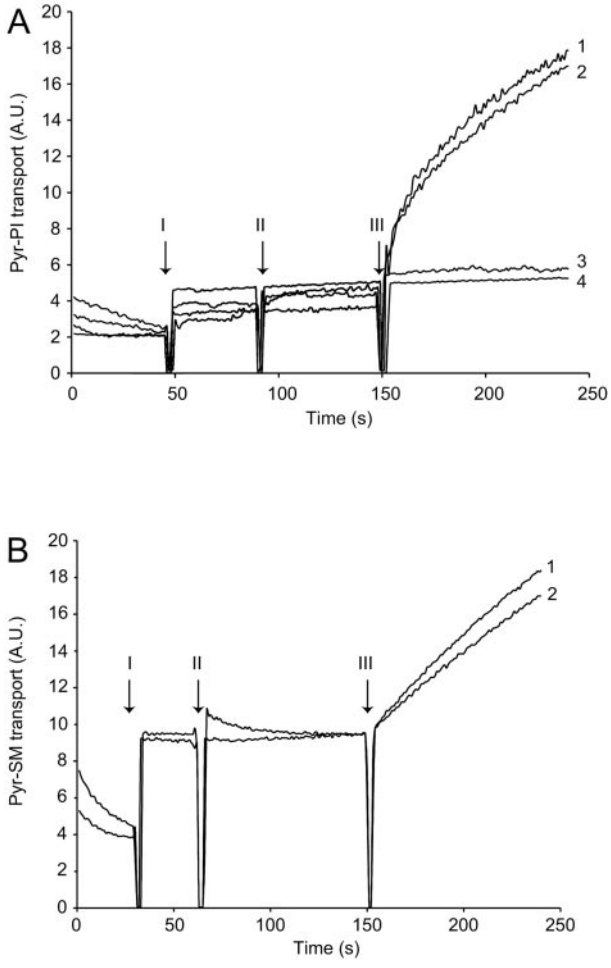

FIG. 4. ***In vitro* phospholipid transfer activity of PI-TPβ.** As described under "Experimental Procedures," the quenched donor vesicles containing pyrene-labeled phospholipids were added to the cuvette followed by the acceptor vesicles (*arrow I*), bovine serum albumin (*arrow II*), and the different PI-TPβs (*arrow III*), and the phospholipid transfer was recorded. *Panel A*, pyrene-PI transfer activity of wtPI-TPβ and the PI-TPβ mutants. (Equal amounts of the 17,500 × *g* bacterial supernatant fraction containing ~5 μg of PI-TPβ were tested). *Trace 1*, PI-TPβ(S262A); *trace 2*, wtPI-TPβ; *trace 3*, PI-TPβ(S165A); *trace 4*, PI-TPβ(S165A/S262A). *Panel B*, pyrene-SM transfer activity of *in vitro* phosphorylated (*trace 2*) and non-phosphorylated (*trace 1*) recombinant PI-TPβ (2 μg).

To estimate the extent of phosphorylation of PI-TPβ, cell lysates were subjected to two-dimensional PAGE analysis. In some experiments GF 109203X was added to the cells for 16 h prior to lysis. Cells were lysed in 400 μl of 20 mM Tris-HCl, pH 8.0, containing 1% Triton X-100. After centrifugation at 17.500 × *g* for 10 min at 4 °C, 100 μg of supernatant protein was precipitated using the Two-Dimensional Clean-Up kit (Amersham Biosciences) according to the manufacturer's instructions. The protein pellets were solubilized in 150 μl of sample buffer (7.7 M urea, 2.2 M thiourea, 2% Triton X-100, 2% CHAPS, 50 mM dithiothreitol, 0.2% carrier ampholytes (pH 3–10), and 0.0002% bromphenol blue) and run on 7-cm immobilized pH gradient strips, pH 5–8, (Bio-Rad) for a total of 24,000 V-h. The strips were equilibrated for 15 min in 6 M urea, 50 mM Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, and 2% (w/v) dithiothreitol and for an additional 15 min in the same solution except that dithiothreitol was replaced with 2.5% iodoacetamide. Finally, the strips were run in the second dimension on a 10% SDS-PAGE Mini Protean 3 gel (Bio-Rad) and further analyzed by Western blotting using the PI-TPβ-specific antibody.

Immunolocalization—The localization of PI-TPβ was determined by indirect immunofluorescence using a polyclonal PI-TPβ-specific antibody. Cells were grown on glass coverslips. In some experiments the

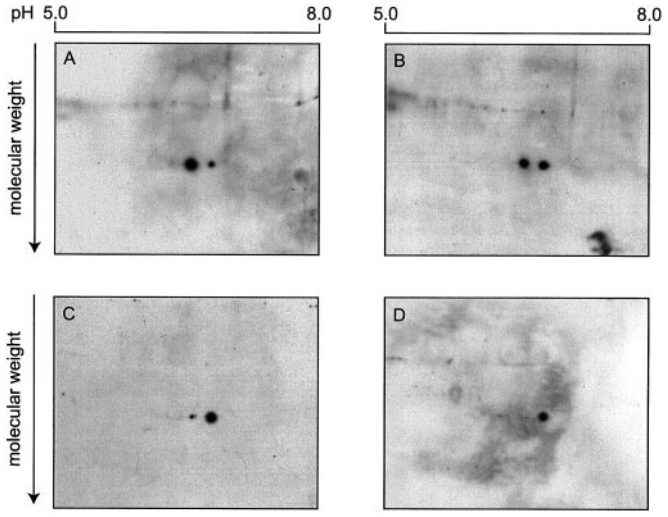

FIG. 5. **Identification of PI-TPβ and its phosphorylated form in SPIβ and SPIβ(S262A) cells.** Cell lysate protein (aliquots of 100 μg) from SPIβ cells (*panel A*), SPIβ cells incubated for 16 h with 5 and 10 μM GF 109203X (*panels B* and *C*), and SPIβ(S262A)6 cells (*panel D*) were subjected to two-dimensional PAGE followed by Western blotting using a PI-TPβ-specific antibody. In the first dimension an immobilized pH gradient strip (pH 5–8) was used. The Western blot of the entire two-dimensional gel is shown. Phosphorylated PI-TPβ runs at pH 6.2 and its non-phosphorylated form at pH 6.5. For further details, see "Experimental Procedures."

cells were incubated for 16 h with GF 109203X (5 μM). The cells were fixed with methanol at −20 °C for 2 min. All further incubations were carried out at room temperature. The cells were rinsed with PBS and incubated with 0.2% gelatin in PBS (PBG) for 1 h to block nonspecific binding sites. The cells were incubated with the PI-TPβ-specific antibody (diluted 1:150 with PBG) for 1 h. The cells were rinsed with PBG, incubated for 1 h with goat anti-rabbit-Cy3 (diluted 1:800 with PBG), rinsed with PBS, and mounted in Mowiol. The labeled cells were viewed with a Leitz inverted microscope.

^{3}H Labeling of SM and Degradation by bSMase—Labeling of the cellular SM pool and degradation by bSMase were performed as described in Ref. 14. Briefly, cells were seeded at 0.8×10^5 cells/55 cm^2 (wtNIH3T3 cells) and 1.0×10^5 cells/55 cm^2 (SPIβ and SPIβ(S262A) cells) in order to obtain identical cell densities at the time of the experiment. After 2 days of growth, the medium was removed, and the cells were labeled for 60 h with 0.5 μCi/ml [*methyl*-^{3}H]choline chloride in Dulbecco's modified Eagle's medium containing 10% newborn calf serum. The cells were washed once with PBS and chased for 2 h with fresh medium. After washing with PBS again, the cells were treated with 200 milliunits/ml bSMase for 30 min. The cells were washed twice with PBS to remove the bSMase and subsequently incubated in fresh medium for 6 h. The cells were harvested by scraping in PBS and sedimented by centrifugation at $350 \times g$ for 5 min. Cell pellets were resuspended in distilled water. An aliquot of the lysate was used for protein determination, and the remainder was used for lipid extraction according to the method of Bligh and Dyer (25). SM was separated from the other lipids by TLC performed in chloroform/methanol/acetic acid/H_2O (50:30:8:5, v/v/v/v). The amount of [^{3}H]SM was determined by scanning the plate with a Berthold Tracemaster 20 automatic TLC linear analyzer.

RESULTS

Determination of the Phosphorylation Sites—Prediction analysis of the PI-TPβ amino acid sequence indicated the presence of eight putative sites for PKC-dependent phosphorylation. These consensus sites contained six threonine (Thr^{58}, Thr^{132}, Thr^{168}, Thr^{197}, Thr^{250}, and Thr^{256}) and two serine (Ser^{165} and Ser^{262}) residues. Phosphoamino acid analysis of *in vitro* phosphorylated His$_6$-tagged wtPI-TPβ demonstrated that this protein was exclusively phosphorylated on a serine residue (Fig. 1).

To establish which serine residues were phosphorylated by PKC, mutants of PI-TPβ were generated in which either Ser^{165} or Ser^{262} was replaced with an alanine residue. A double mutant in which both Ser^{165} and Ser^{262} were replaced was also prepared. His$_6$-tagged-wtPI-TPβ, -PI-TPβ(S165A), -PI-TPβ(S262A), and -PI-TPβ(S165A/S262A) purified by affinity chromatography were phosphorylated by PKC at two different protein concentrations (Fig. 2*A*). As shown in Fig. 2*B*, wtPI-TPβ was a substrate for PKC (*lanes 1–4*). Phosphorylation was strongly reduced in the absence of Ca^{2+} and PS (*lanes 1* and *3*). Phosphorylation of PI-TPβ(S165A) was comparable with that of wtPI-TPβ, indicating that Ser^{165} had little or no phosphorylation by PKC (*lanes 5–8*). PI-TPβ(S262A) was a bad substrate for PKC, indicating that Ser^{262} was the major site of phosphorylation (*lanes 9–12*). The double mutant PI-TPβ(S165A/S262A) was not phosphorylated (*lanes 13–16*). These data show that PI-TPβ contains two phosphorylation sites of which Ser^{262} is the major and Ser^{165} the minor site.

Phosphopeptide Mapping—Two-dimensional analysis of ^{32}P-labeled peptides derived from a tryptic digest of wtPI-TPβ showed three major and several minor spots (Fig. 3, *left panel*). The tryptic peptide map of ^{32}P-labeled PI-TPβ(S165A) yielded the same three major spots (Fig. 3, *middle panel*), strongly suggesting that all three spots contained phosphorylated Ser^{262} as a result of partial cleavage of the bonds in the sequence Arg-Lys-Lys-Gly-Ser^{262}-Val-Arg. This was confirmed by the tryptic peptide map of PI-TPβ(S262A), which lacked these three spots (Fig. 3, *right panel*). Instead, phosphorylation of PI-TPβ(S262A) yielded one spot representing the peptide containing ^{32}P-labeled Ser^{165}. This labeled peptide (indicated by the *arrowheads* in Fig. 3) was absent from the phosphopeptide map of PI-TPβ(S165A) but was present in the phosphopeptide map wtPI-TPβ as a very minor spot. This indicates that the phosphorylation of wtPI-TPβ is almost exclusively restricted to Ser^{262}.

Phospholipid Transfer Activity of wtPI-TPβ and the PI-TPβ Mutants—The PI transfer activity of the PI-TPβs was determined in the continuous fluorescent phospholipid transfer assay. As shown in Fig. 4*A*, PI-TPβ(S262A) and wtPI-TPβ expressed an equal activity toward PI (*curves 1* and *2*). However, when Ser^{165} was replaced with an alanine residue, the ensuing mutants PI-TPβ(S165A) and PI-TPβ(S165A/S262A) were completely inactive in the PI transfer assay (*curves 3–4*). The PC and SM transfer activities of the PI-TPβs were also determined, and the results were comparable with those observed for the PI transfer activity (data not shown).

To establish whether phosphorylation had an effect on transfer activity, wtPI-TPβ was phosphorylated by PKC under optimized conditions (1 mM ATP, 30 min of incubation), yielding a stoichiometry of 0.5 mol of phosphate/mol PI-TPβ. Under these conditions the SM transfer activity was not affected (Fig. 4*B*). Phosphorylation of PI-TPβ also had no effect on the PC and PI transfer activities (data not shown).

Overexpression of PI-TPβ(S262A) in NIH3T3 Cells—To establish the effect of the major site of phosphorylation on the cellular function of PI-TPβ, mouse NIH3T3 cells were transfected with the pBK-CMV-PI-TPβ(S262A) construct. Stable cell lines were selected using the antibiotic G418; from the G418-resistant clones that appeared after 3 weeks of selection, 7 cell lines were analyzed by Western blotting. Two cell lines, designated as SPIβ(S262A)6 and SPIβ(S262A)7, were selected for further experiments. By scanning the immunoblot, it was estimated that SPIβ(S262A)6 and SPIβ(S262A)7 contained 9.0 ± 0.7 and 8.1 ± 1.3 ng of PI-TPβ(S262A), respectively, per 100 μg of cytosolic protein. For comparison, wtNIH3T3 cells contained 1.0 ± 0.3 ng of PI-TPβ, and NIH3T3 cells overexpressing wtPI-TPβ (SPIβ) contained 10.6 ± 0.3 ng of PI-TPβ (14).

In Situ Phosphorylation of PI-TPβ—Lysates from SPIβ cells

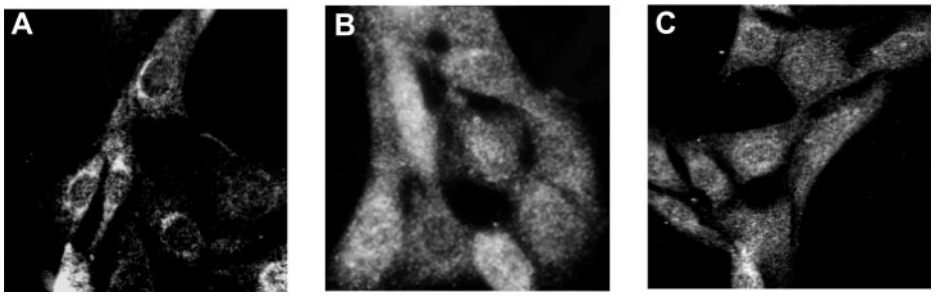

FIG. 6. **Intracellular localization of wtPI-TPβ and PI-TPβ(S262A).** The localization of PI-TPβ was determined in SPIβ (*panel A*), SPIβ cells incubated for 16 h with 5 μM GF 109203X (*panel B*), and SPIβ(S262A)6 cells (*panel C*) by indirect immunofluorescence using a PI-TPβ-specific antibody. SPIβ(S262A) clones 6 and 7 gave identical results.

were analyzed by two-dimensional PAGE and Western blotting using an anti-PI-TPβ antibody. In initial experiments, immobilized pH gradient strips of pH 3–10 were used showing two spots of PI-TPβ (estimated pH 6.5) that were poorly separated. To improve resolution, further analysis was carried out using strips of pH 5–8. As shown in Fig. 5*A*, PI-TPβ collected in a minor spot at pH 6.5 and a major spot at pH 6.2. Incubation of the SPIβ cells with the PKC inhibitor GF 109203X (5 μM) resulted in a shift of PI-TPβ from the pH 6.2 to the pH 6.5 form (Fig. 5*B*). The shift to the pH 6.5 form was virtually complete when the cells were incubated with 10 μM PKC inhibitor (Fig. 5*C*). From this we infer that the pH 6.2 spot represents the phosphorylated form. In support of this, a similar analysis of lysates from SPIβ(S262A) cells demonstrated that the mutated PI-TPβ collected exclusively at pH 6.5 (Fig. 5*D*). These data indicate that *in situ* PI-TPβ is phosphorylated at Ser^{262} and that this phosphorylation is dependent on PKC. From densitometric analysis we estimate that *in situ* PI-TPβ is at least 85% phosphorylated. Addition of the antibody to the cell lysate followed by incubation with protein A linked to beads failed to immunoprecipitate PI-TPβ. Hence, immunoprecipitation of PI-TPβ from cells labeled with inorganic ^{32}P could not be carried out.

Ser^{262} and the Golgi Localization of PI-TPβ—In a previous study it was shown that PI-TPβ in Swiss mouse 3T3 fibroblasts was mainly associated with the perinuclear Golgi (7, 8). The Golgi localization of PI-TPβ was confirmed in SPIβ cells by indirect immunofluorescence using a specific anti-PI-TPβ antibody (Fig. 6, *panel A*). Because of the very low amount of endogenous PI-TPβ, this Golgi labeling was hardly visible in NIH3T3cells (data not shown). Incubation of SPIβ cells with the PKC inhibitor GF 109203X resulted in a redistribution of PI-TPβ throughout the cell (*panel B*), strongly suggesting that phosphorylation of Ser^{262} was a prerequisite for Golgi localization. This was confirmed by showing that PI-TPβ(S262A) was also present throughout the cytoplasm (*panel C*).

Effect of PI-TPβ(S262A) Overexpression on SM Synthesis—Previously it was shown that plasma membrane levels of SM were maintained in SPIβ cells under conditions in which SM was hydrolyzed to ceramide by exogenous sphingomyelinase (14). This was not observed in wtNIH3T3 cells, which suggested that a certain level of PI-TPβ was required for maintaining the steady-state SM level. In order to investigate whether the Golgi localization of PI-TPβ played a role in the rapid conversion of ceramide into SM, the above experiment was repeated with the SPIβ(S262A) cell lines. Cells were incubated with [^{3}H]choline to label cellular SM and subjected to treatment with bSMase. After 30 min of SM degradation, the bSMase was removed, and the cells were allowed to recover in fresh medium for 6 h. After the incubation with bSMase, the hydrolysis of SM amounted to ~35% in NIH3T3, SPIβ, and SPIβ(S262A) cells (Fig. 7). At the end of the 6-h recovery

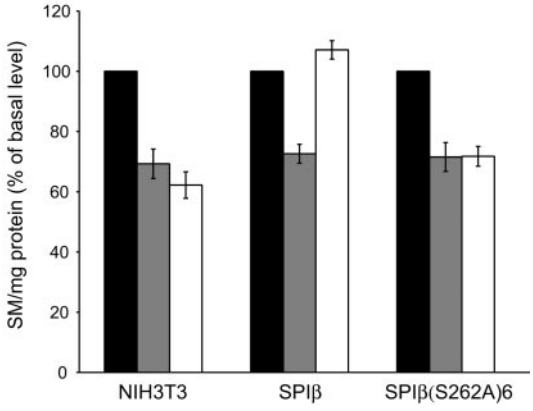

FIG. 7. **SM synthesis in NIH3T3, SPIβ, and SPIβ(S262A) cells.** [^{3}H]Choline-labeled cells were incubated with 200 milliunits/ml bSMase. After 30 min of treatment, the cells were washed with PBS and incubated in fresh medium for 6 h. Resynthesis of SM was determined as described under "Experimental Procedures." The extent of resynthesis is expressed as the percentage of [^{3}H]SM/mg of cellular protein relative to untreated cells. Values are the means ± S.D. of three independent experiments performed in duplicate. *Black bars*, untreated cells; *gray bars*, bSMase-treated cells; *open bars*, 6 h after bSMase treatment. Similar results were obtained for SPIβ(S262A) clones 6 and 7

period, SM levels were not restored in NIH3T3 and the SPIβ(S262A) cells, whereas SM was restored to basal levels in SPIβ cells. Given that the *in vitro* SM transfer activity of PI-TPβ(S262A) was normal, these results strongly suggest that the Golgi localization is required for PI-TPβ to be able to stimulate SM resynthesis.

DISCUSSION

In this study we have shown that PI-TPβ is a substrate for PKC. By mutation analysis we could establish that Ser^{262} was the main phosphorylation site. However, Ser^{165}, which is analogous to Ser^{166} in PI-TPα, was also phosphorylated although to a very limited extent. In a previous study we had shown that the PKC-dependent phosphorylation of PI-TPα was restricted to Ser^{166} (18). The PI-TPα isoform, which is highly homologous to PI-TPβ (similarity of 94%), lacks Ser^{262} (9). Assuming that phosphorylation of the PI-TP isoforms is important for the regulation of their function, we were interested to establish the role of Ser^{262} in the function of PI-TPβ.

The peptide maps of wtPI-TPβ and PI-TPβ(S165A) show three major spots, which are absent from the map of PI-TPβ(S262A) (Fig. 3). This indicates that these three spots represent peptides containing phosphorylated Ser^{262}. The formation of these peptides is probably due to the presence of multiple tryptic cleavage sites in the amino acid sequence of the peptide Met-Arg-Lys-Lys-Gly-Ser^{262}-Val-Arg. Partial digestion would yield the peptides Lys-Lys-Gly-Ser-Val-Arg, Lys-

Gly-Ser-Val-Arg, and Gly-Ser-Val-Arg. According to the method described in Ref. 22, we could assign Lys-Lys-Gly-Ser-Val-Arg to spot 1, Lys-Gly-Ser-Val-Arg to spot 2, and Gly-Ser-Val-Arg to spot 3. The tryptic map of PI-TPβ(S262A) showed one spot representing a peptide containing phosphorylated Ser^{165}. This spot was barely visible in the peptide map of wtPI-TPβ, indicating that phosphorylation was almost exclusively restricted to Ser^{262}.

Replacement of Ser^{165} with Ala yielded PI-TPβ(S165A) and the double mutated PI-TPβ(S165A/S262A), both of which *in vitro* completely lacked PI, PC, and SM transfer activity. On the other hand, PI-TPβ(S262A) was fully active. Mutation of the corresponding serine (Ser^{166}) in PI-TPα also yielded an inactive protein (18). From the three-dimensional structure it can be inferred that Ser^{166} is exposed at the surface as part of the regulatory loop of PI-TPα (26). Hence it is possible that the loss of transfer activity is due to the inability of PI-TPα(S166A) to properly interact with the membrane interface. However, at this stage we cannot exclude the possibility that replacement of Ser^{166} with Ala affects the proper folding of the protein during synthesis in *E. coli*. The same explanations may hold for the lack of transfer activity observed in PI-TPβ(S165A) and PI-TPβ(S165A/S262A). After PKC-dependent phosphorylation, the phospholipid transfer activity of PI-TPβ was unchanged, indicating that phosphorylation of Ser^{262} had no effect (Fig. 4).

The lysates from SPIβ cells contained two forms of PI-TPβ that could be separated by isoelectric focusing. From densitometric analysis it was estimated that 85% of the PI-TPβ collected at pH 6.2 and 15% at pH 6.5. Treatment of the cells with the PKC inhibitor GF 109203X shifted PI-TPβ to pH 6.5, strongly suggesting that the spot at pH 6.2 represented the phosphorylated form of PI-TPβ. Because the lysate from the SPIβ(S262A) cells contained predominantly PI-TPβ at pH 6.5, we conclude that *in situ* PI-TPβ is constitutively phosphorylated at Ser^{262}. Given that GF 109203X inhibits conventional and novel type PKCs (27, 28), we do not know which PKC isoform is involved in the phosphorylation of PI-TPβ. Because it is unlikely that PKC is constitutively active in these cells, it appears that phosphorylated PI-TPβ in association with the Golgi is a poor substrate for protein phosphatase. In a previous study PI-TPβ isolated from bovine brain could not be phosphorylated by PKC (8). Because bovine brain protein is 99% identical to murine PI-TPβ, we consider it likely that in this case also PI-TPβ is mainly present in its phosphorylated form.

It has previously been reported that in Swiss mouse 3T3 fibroblasts, PI-TPβ was predominantly associated with the Golgi (8). In the present study we have confirmed that PI-TPβ was associated with the Golgi complex in the SPIβ cells (Fig. 6, *panel A*). By incubating SPIβ cells with GF 109203X, a relocation of PI-TPβ from the Golgi to the cytoplasm was observed (*panel B*). A similar distribution throughout the cytoplasm was observed for PI-TPβ(S262A) expressed in NIH3T3 cells (*panel C*). These observations indicate that Ser^{262} has to be phosphorylated for PI-TPβ to be associated with the Golgi system. It is to be noted that the phosphorylation site Ser^{262} is only present in PI-TPβ, whereas the phosphorylation site $Ser^{165/166}$ is conserved in all PI-TPs identified so far, with the exception of PI-TP from *Caenorhabditis elegans* (26).

In contrast to wtPI-TPβ, PI-TPβ(S262A) that is overexpressed in mouse fibroblasts is not able to stimulate the resynthesis of SM after the breakdown of this lipid by sphingomyelinase. Because the mutant protein expresses full lipid transfer activity *in vitro*, we infer that the association of PI-TPβ with the Golgi is a prerequisite for PI-TPβ to stimulate rapid SM replenishment. SM and cholesterol regulation in the Golgi has also been linked to the Golgi localization and phosphorylation of the oxysterol-binding protein (29). Similar to its yeast analog Sec14p, PI-TPβ may play a role in the budding of SM-containing vesicles from the Golgi (10, 30). It has been well established that the intracellular transport of SM is linked to the assembly and dynamics of lipid rafts (31). We are currently investigating whether PI-TPβ is involved in this process.

REFERENCES

1. Wirtz, K. W. A. (1997) *Biochem. J.* **324,** 353–360
2. Wirtz, K. W. A. (1991) *Annu. Rev. Biochem.* **60,** 73–99
3. van Paridon, P. A., Visser, A. J., and Wirtz, K. W. A. (1987) *Biochim. Biophys. Acta* **898,** 172–180
4. Helmkamp Jr., G. M., Harvey, M. S., Wirtz, K. W. A., and Van Deenen, L. L. M. (1974) *J. Biol. Chem.* **249,** 6382–6389
5. Helmkamp Jr., G. M. (1985) *Chem. Phys. Lipids* **38,** 3–16
6. Helmkamp Jr., G. M. (1986) *J. Bioenerg. Biomembr.* **18,** 71–91
7. de Vries, K. J., Westerman, J., Bastiaens, P. I., Jovin, T. M., Wirtz, K. W. A., and Snoek, G. T. (1996) *Exp. Cell Res.* **227,** 33–39
8. de Vries, K. J., Heinrichs, A. A., Cunningham, E., Brunink, F., Westerman, J., Somerharju, P. J., Cockcroft, S., Wirtz, K. W. A., and Snoek, G. T. (1995) *Biochem. J.* **310,** 643–649
9. Tanaka, S., and Hosaka, K. (1994) *J. Biochem.* (*Tokyo*) **115,** 981–984
10. Ohashi, M., de Vries, K. J., Frank, R., Snoek, G. T., Bankaitis, V., Wirtz, K. W. A., and Huttner, W. B. (1995) *Nature* **377,** 544–547
11. Fensome, A., Cunningham, E., Prosser, S., Tan, S. K., Swigart, P., Thomas, G., Hsuan, J., and Cockcroft, S. (1996) *Curr. Biol.* **6,** 730–738
12. Cunningham, E., Tan, S. K., Swigart, P., Hsuan, J., Bankaitis, V., and Cockcroft, S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6589–6593
13. Snoek, G. T., Berrie, C. P., Geijtenbeek, T. B. H., van der Helm, H. A., Cadee, J. A., Iurisci, C., Corda, D., and Wirtz, K. W. A. (1999) *J. Biol. Chem.* **274,** 35393–35399
14. van Tiel, C. M., Luberto, C., Snoek, G. T., Hannun, Y. A., and Wirtz, K. W. A. (2000) *Biochem. J.* **346,** 537–543
15. Bankaitis, V. A. (2002) *Science* **295,** 290–291
16. Alb, J. G., Phillips, S. E., Rostand, K., Cui, X., Pinxteren, J., Cotlin, L., Manning, T., Guo, S., York, J. D., Sontheimer, H., Collawn, J. F., and Bankaitis, V. A. (2002) *Mol. Biol. Cell* **13,** 739–754
17. Snoek, G. T., Westerman, J., Wouters, F. S., and Wirtz, K. W. A. (1993) *Biochem. J.* **291,** 649–656
18. van Tiel, C. M., Westerman, J., Paasman, M., Wirtz, K. W. A., and Snoek, G. T. (2000) *J. Biol. Chem.* **275,** 21532–21538
19. Geijtenbeek, T. B., de Groot, E., van Baal, J., Brunink, F., Westerman, J., Snoek, G. T., and Wirtz, K. W. A. (1994) *Biochim. Biophys. Acta* **1213,** 309–318
20. Westerman, J., de Vries, K. J., Somerharju, P., Timmermans-Hereijgers, J. L., Snoek, G. T., and Wirtz, K. W. A. (1995) *J. Biol. Chem.* **270,** 14263–14266
21. Huang, K. P., Chan, K. F., Singh, T. J., Nakabayashi, H., and Huang, F. L. (1986) *J. Biol. Chem.* **261,** 12134–12140
22. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) *Methods Enzymol.* **201,** 110–149
23. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
25. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
26. Yoder M. D., T. L. M., Tremblay J. M., Oliver R. L., Yarbrough L. R., and Helmkamp Jr., G. M. (2001) *J. Biol. Chem.* **276,** 9246–9252
27. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charons, D., and Kirilovsky, J. (1991) *J. Biol. Chem.* **266,** 15771–15781
28. Mwanjewe, J., Spitaler, M., Ebner, M., Windegger, M., Geiger, M., Kampfer, S., Hofmann, J., Uberall, F., and Grunicke, H. H. (2001) *Biochem. J.* **359,** 211–217
29. Ridgway, N. D., Lagace, T. A., Cook, H. W., and Byers, D. M. (1998) *J. Biol. Chem.* **273,** 31621–31628
30. Bankaitis, V. A., Malehorn, D. E., Emr, S. D., and Greene, R. (1989) *J. Cell Biol.* **108,** 1271–1281
31. Simons, K., and Toomre, D. (2000) *Nat. Rev. Mol. Cell. Biol.* **1,** 31–39