Identification and Characterization of the Autophosphorylation Sites of Phosphoinositide 3-Kinase Isoforms β and γ

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Class I phosphoinositide 3-kinases (PI3Ks) are bifunctional enzymes possessing lipid kinase activity and the capacity to phosphorylate their catalytic and/or regulatory subunits. In this study, in vitro autophosphorylation of the G protein-sensitive p85-coupled class Iα PI3Kβ and p101-coupled class Iα PI3Kγ was examined. Autophosphorylation sites of both PI3K isoforms were mapped to C-terminal serine residues of the catalytic p110 subunit (i.e. serine 1083 of p110β and serine 1011 of p110γ). Like other class Iα PI3K isoforms, autophosphorylation of p110γ resulted in down-regulated PI3Kβ lipid kinase activity. However, no inhibitory effect of p110γ autophosphorylation on PI3Kγ lipid kinase activity was observed. Moreover, PI3Kβ and PI3Kγ differed in the regulation of their autophosphorylation. Whereas p110β autophosphorylation was stimulated neither by Gβγ complexes nor by a phosphotyrosinyl peptide derived from the platelet-derived growth factor receptor, autophosphorylation of p110γ was significantly enhanced by Gβγ in a time- and concentration-dependent manner. In summary, we show that autophosphorylation of both PI3Kβ and PI3Kγ occurs in a C-terminal region of the catalytic p110 subunit but differs in its regulation and possible functional consequences, suggesting distinct roles of autophosphorylation of PI3Kβ and PI3Kγ.

Class I phosphoinositide 3-kinases (PI3Ks) are lipid kinases that are activated in response to a variety of extracellular stimuli including hormones, neurotransmitters, and growth factors, which act via G protein-coupled receptors or receptor tyrosine kinases. These lipid kinases phosphorylate the D3 position of the inositol ring of phosphoinositides, thus generating intracellular lipid second messengers (1, 2). PtdIns(4,5)-P2 is in vitro substrate of class I PI3Ks, although these enzymes predominantly produce PtdIns(3,4,5)-P3 in vivo. Class I PI3K lipid products transmit signals by recruiting intracellular effector molecules to the membrane, which contain particular pleckstrin homology domain modules. Effectors include serine/threonine kinases like Akt/protein kinase B, Tec family tyrosine kinases, and guanine nucleotide exchange factors for monomeric GTP-binding proteins like Grp1 and Vav (3). Consequently, class I PI3Ks are involved in the regulation of a wide variety of cellular functions such as differentiation, proliferation, survival, migration, and metabolism (4, 5).

All class I PI3Ks are heterodimers consisting of a p110 catalytic and a p85 or p101 type regulatory subunit. According to the type of their associated regulatory subunit, class I PI3Ks can be further distinguished. The class Iα catalytic p110α, -β, and -γ subunits complex with adaptor molecules containing two Src homology 2 domains, of which p85 is the prototype. Through interaction of the adaptor Src homology 2 domains with phosphotyrosines, class Iα PI3Ks are activated by receptor tyrosine kinases. In contrast, the only class Iγ member, p110γ, associates with a p101 regulatory subunit and is not recruited to tyrosine-phosphorylated receptor tyrosine kinases. PI3Kγ is regulated by G protein-coupled receptors through direct interaction with Gβγ complexes of heterotrimeric G proteins (6–9). Furthermore, class Iγ PI3Kγ is also sensitive to Gβγ and thus may function as a coincidence detector integrating tyrosine kinase- and G protein-dependent signals (10–12).

In addition to their lipid kinase activity, all class I PI3Ks possess an intrinsic protein kinase activity in vitro (13). This enzymatic quality was first characterized as autophosphorylation of catalytic and regulatory subunits. PI3Ks phosphorylates its p85 adaptor subunit at serine 608, whereas autophosphorylation of PI3Kγ occurs at serine 1039 of the catalytic p110γ subunit. Both phosphorylations result in down-regulation of the lipid kinase activity (14–16). Moreover, in Jurkat T cells, p110γ phosphorylation was stimulated by CD28 in vivo (15). There is some in vitro evidence that class Iγ PI3Ks can phosphorylate other substrates such as the insulin receptor substrate-1 adaptor protein and PDE3B, but the physiological significance of these phosphorylations remains unknown (17–21). Recently it was reported that a "protein kinase-only" mutant of the G-protein-regulated PI3Kγ still activated mitogen-activated protein kinase

* This work was supported by grants from the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie (DFG Nu53-6/1; SFB518). 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.

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§ The abbreviations used are: PI3K, phosphoinositide 3-kinase; p110, catalytic subunit of PI3Ks; p101, subunit associated with p110γ; p85, regulatory subunit of class Iα PI3Ks; PtdIns, phosphatidylinositol; PtdIns(4-5)-P, phosphatidylinositol 4,5-phosphate; PtdIns(3,4,5)-P3, phosphatidylinositol 3,4,5-trisphosphate; GST, glutathione S-transferase; Hs, human/histidine tag; MS, mass spectrometry; MALDI-MS, matrix-assisted laser desorption/ionization MS; ES, ESI-MS, electrospray ionization tandem MS; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography; PKO, protein kinase-only; TOF, time of flight.

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pathways in cells, whereas no activation of Akt/protein kinase B by this mutant occurred (22).

Autophosphorylation of both catalytic and regulatory subunits of PI3Kβ and PI3Ky has been proposed, but many questions regarding the autophosphorylation sites and the functional relevance of these autophosphorylation events remain unanswered. Therefore, in the present study, we examined the in vitro autophosphorylation of the G protein-sensitive class I PI3Kβ and PI3Ky. Recombinant heterodimeric PI3Ks were purified and analyzed for their autophosphorylation. Phosphorylated amino acids were identified, and the effect of autophosphorylation on PI3K lipid kinase activity was studied. Interestingly, we observed significant differences in the regulation and functional consequences of the autophosphorylation of PI3Kβ and PI3Ky.

EXPERIMENTAL PROCEDURES

Recombinant PI3Ks—Construction and characterization of recombinant baculoviruses for expression of GST-p110α, p110γ, GST-p110α, and p85α were described previously (6–8). A pFastBacHTa baculovirus transfer vector (Invitrogen) was used to generate His-p110β and His-p110γ full-length constructs using NotI/SalI and NeotamHI cloning sites, respectively. Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Oligonucleotides for generation of p110αS1070 mutants (mutated residues are underlined) were as follows: 5′-GGA AAG ACT ACA GAG CTT AAG CTG CAG TGG TCG-3′ and 5′-CGA CTG CAG CTT AAG CTG TCT ACT AGT CTT-3′ (for S1070A), 5′-GGA AAG ACT ACA GAG CTT AAG CTG CAG TGG TCG-3′ and 5′-CGA CTG CAG CTT AAG CTG TCT ACT AGT CTT-3′ (for S1070D), and 5′-GGA AAG ACT ACA GAG CTT AAG CTG CAG TGG TCG-3′ and 5′-CGA CTG CAG CTT AAG CTG TCT ACT AGT CTT-3′ (for S1070E).

Oligonucleotides used to create the p110αS1101 mutants were as follows: 5′-GGA AAG ACT ACA GAG CTT AAC CAT GAA GCC TAA TCT ATT AGG CTA GAA TCA-3′ and 5′-GAT TAC GCC GCA TCA-5′ (for S1101E).

Recombinant viruses expressing hexahistidine-tagged p110α, p110γ, and mutants thereof were generated using the Bac-to-Bac Expression System (Invitrogen) following the manufacturer's instructions. Expression of PI3K isoforms was carried out according to published protocols (11) with the exception that the partially purified proteins were subjected to an additional chromatographic step on a 1 ml Resource Q fast protein liquid chromatography column (Amersham Biosciences). For that purpose, proteins were diluted in buffer A (20 mM Tris/HCl, pH 8.0, 10 mM MgCl2, 100 mM NaCl, 40 mM HEPES, pH 7.4, 1 mM diethiothreitol, 1 mM β-glycerophosphate (vesicle buffer) with some modifications. Lipid vesicles (50 ml containing 320 mM phosphatidylethanolamine, 140 μM phosphatidylinositol, 30 μM sphingomyelin, and 40 μM PtdIns-4,5-P2, in vesicle buffer) were mixed with stimuli as indicated and incubated on ice for 10 min. It should be noted that we ensured that the effects of Gβγ on PI3K activity were determined basically as described previously (11). In brief, assays were conducted in a final volume of 50 μl containing 0.1% bovine serum albumin, 1 mM EGTA, 0.2 mM EDTA, 7 mM MgCl2, 100 mM NaCl, 40 mM HEPES, pH 7.4, 1 mM diethiothreitol, 1 mM β-glycerophosphate (vesicle buffer) with some modifications. Lipid vesicles (50 ml containing 320 mM phosphatidylethanolamine, 140 μM phosphatidylinositol, 30 μM sphingomyelin, and 40 μM PtdIns-4,5-P2, in vesicle buffer) were mixed with stimuli as indicated and incubated on ice for 10 min.

Gβγ Complexes and Peptides—Expression and purification of recombinant Gβγ heterodimers, complexes was carried out as published (11). Purified proteins were quantified by Coomassie Blue staining following SDS-PAGE and transferred to nitrocellulose membranes. Dried membranes were exposed to Fuji imaging plates, and autoradiographic signals were quantitated with a BAS 1500 Fuji Imager (Raytest, Straubenhardt, Germany).

Cell Culture, Transfection, and Preparation of Cell Lysates—HEK293 cells (American Type Culture Collection, Manassas, VA) were grown in minimal essential medium with Earle's salts supplemented with 10% fetal calf serum and antibiotics. Subconfluent cultures were transfected in 3-cm dishes with pcDNA3-MLP receptor (0.2 μg), pcDNA3-p101 (0.4 μg), and pcDNA3-p110γ (0.4 μg) variants, using the FuGene 6 transfection reagent (Roche Molecular Biochemicals) following the manufacturer's instructions. For preparation of whole cell lysates, cells were directly lysed in sample buffer according to Laemmli. (39).
MS/MS fragment ions of the p110β C-terminal phosphopeptide

| m/z | Loss of neutral H$_2$PO$_4^-$ |
|-----|----------------------------|
| y$^+$ ion | 505.18, 407.20 |
| y$^+$ ion | 1003.47, 905.50 |
| y$^+$ ion | 1104.52, 1006.54 |
| y$^+$ ion | 1241.58 |
| b$_1$ ion | 209.10 |
| b$_2$ ion | 310.15 |
| b$_3$ ion | 565.32 |
| b$_4$ ion | 693.42 |
| b$_5$ ion | 808.44 |
| b$_6$ ion | 971.51 |
| b$_7$ ion | 1127.61 |
| b$_8$ ion | 1294.61 |

**FIG. 2.** p110β autophosphorylation at a C-terminal serine residue. A, heterodimeric recombinant PI3Kβ purified from S9 cells was subjected to SDS-PAGE and visualized by Coomasie staining. Apparent molecular masses (kDa) of marker proteins are indicated. B, peptide mass fingerprint analysis of p110β. Autophosphorylated p110β was digested in gel using cyanogen bromide, the resulting peptides were separated by reversed-phase HPLC, and the fractions were analyzed by MALDI-MS. The peak with m/z 1312.65 (calculated m/z 1312.62) corresponds to the phosphopeptide sequence T061-AHTVRKDYP~S1070~. C, p110β in which serine 1070 was mutated to alanine shows a loss of autophosphorylation activity. Heterodimeric PI3Kβ either containing wild-type p110β (WT) or a p110β mutant (S1070A) was subjected to a protein kinase assay in the presence of Mn$^{2+}$. Shown are one representative autoradiograph and the corresponding Coomasie-stained protein bands as loading control. D, lipid kinase activity of mutant p110β. Equal amounts of purified heterodimeric PI3Kβ (His-p110β/His-p85β) either containing wild-type p110β (WT) or a p110β mutant (S1070D, S1070E, or S1070A) were tested for their enzymatic activity in a lipid kinase assay in the absence (−) and presence (+) of 120 nM purified Gβγ, 100 nM tyrosine-phosphorylated peptide, and both stimuli. Experiments were carried out in the presence of Mg$^{2+}$. Indicated are mean values ± S.D. of three independent experiments. E, Mn$^{2+}$-dependent protein kinase activity of PI3Kβ in the presence of increasing amounts of a synthetic peptide derived from the C terminus of p110β. One representative autoradiograph of three independent experiments is shown.

**Phosphoamino Acid Analysis**—Autophosphorylated PI3Kγ was subjected to SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Millipore Corp.), and the phosphorylated p110β band was excised. The protein was hydrolyzed in 6 N HCl for 1 h at 110 °C. The sample was vacuum-dried, and amino acids were resuspended in 5 μl of pH 1.9 buffer (0.078% (v/v) acetic acid, 0.925% (v/v) formic acid) containing 2 μg each of phosphoserine, phosphothreonine, and phosphotyrosine as internal standards. The sample was applied to a cellulose thin layer plate, and electrophoresis was carried out in the first dimension. In the second dimension, an electrophoretic separation was carried out in pH 3.5 buffer (0.05% (v/v) acetic acid, 0.005% (v/v) pyridine, 50 mM EDTA) at 500 V for 20 min. The unlabeled phosphoamino acids were visualized by spraying the plate with 0.2% (w/v) ninhydrin in acetone, and the radio-labeled phosphoamino acids were detected by autoradiography.

**Determination of p110β and p110γ Autophosphorylation Sites**—Gel-excised autophosphorylated p110 spots (100–150 pmol) were washed with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate for 45 min at 55 °C. Alkylation was performed by replacing the dithiothreitol solution with 50 mM iodoacetamide in 100 mM ammonium bicarbonate. Following a 20-min incubation period at 25 °C in the dark, the gel pieces were washed with 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. Gel slices were incubated overnight at 37 °C in 5 mM ammonium bicarbonate, containing 1 μg of chymotrypsin (sequencing grade; Roche Molecular Biochemicals), for p110γ or at room temperature in 50% (v/v) trifluoroacetic acid, containing 10 mg/ml cyanogen bromide, for p110β. To extract the peptides, 0.5% (v/v) trifluoroacetic acid in acetonitrile was added, and the separated liquid was dried under vacuum, redissolved in 5 μl of buffer B (0.1% (v/v) formic acid), and loaded onto a Vydac C18 column (150 x 1.0 mm, 5 μm) for micro-liquid chromatography separation. Elution was performed using a linear gradient of 5%–80% buffer C in 60 min at an eluent flow rate of 30 μl/min. Buffer C was 0.1% (v/v) formic acid in acetonitrile/water (9:2, v/v), containing 0.1% (v/v) formic acid. Fractions were collected, their radioactivity was determined by Cerenkov counting, and phosphopeptides were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). MALDI-MS measurements were performed on a Voyager-DE STR Bio-Spectrometry work station MALDI-TOF mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA) using α-cyano-4-hydroxycinnamic acid as the matrix. The program FindMod (available on the World Wide Web at expasy.ch/tools/findmod) was used to interpret the MS spectra of protein digests. Amino acid sequences of the phosphopeptides were determined by nanoelectrospray tandem mass spectrometry (nanoESI-MS/MS). The liquid chromatography fractions were lyophilized and redissolved in 5 μl of 1% (v/v) formic acid in methanol/water (95:5, v/v). The MS/MS measurements were performed with a nanoelectrospray hybrid quadrupole mass spectrometer Q-TOF (Micromass, Manchester, UK). The collision gas was argon at a pressure of 6.0 × 10⁻¹⁰ mbar in the collision cell.
**RESULTS**

In Vitro Autophosphorylation of Class IA PI3Ks—Class IA PI3Kα and PI3Kβ isoforms phosphorylate either their p85 adaptor subunit and/or the catalytic p110 subunit itself (14, 15). Autophosphorylation of p110β occurs on serine 1039 within the C terminus. Alignment of the C termini of class IA catalytic subunits shows that p110α, which does not autophosphorylate, contains no C-terminal serine, whereas p110β does have one (serine 1070) (Fig. 1A). Recent reports have described autophosphorylation of both subunits of PI3Kβ (i.e. p85 and p110β) (25–27). In order to analyze autophosphorylation of PI3K isoforms in vitro, we expressed recombinant heterodimeric PI3Kα and PI3Kβ in insect cells and measured their protein kinase activities (Fig. 1B). As anticipated, the p85 adaptor of PI3Kα was phosphorylated in the presence of Mn2+ only (Fig. 1B, left panel). In contrast to p110α, p110β autophosphorylated its catalytic subunit. This autophosphorylation of p110β was also largely Mn2+-dependent, since in vitro phosphorylation levels in the presence of Mg2+ reached a maximum of only 5–10% compared with the level observed in the presence of Mn2+ (see Fig. 1B, right panel). Furthermore, in the presence of Mn2+, a small but significant phosphorylation of the p85 subunit of PI3Kβ was evident. These data indicate that both subunits are phosphorylated, with p110β being the main substrate of PI3Kβ autophosphorylation.

**p110β Autophosphorylates a C-terminal Serine Residue**—Speculating that p110β autophosphorylates its C terminus, the in vitro phosphorylated and [32P]phosphate-labeled protein (Fig. 2A) was cleaved with cyanogen bromide in order to generate a C-terminal peptide, which was then analyzed by mass spectrometry. The resulting peptides were separated by reversed-phase HPLC, and the radioactivity of each fraction was determined. The main radioactive fraction was examined by MALDI-MS, and a peptide corresponding to the phosphorylated C terminus of p110β (m/z 1312.65) could be identified (Fig. 2B). Sequencing of this phosphopeptide by nanoESI-MS/MS revealed the 1061AHTVRDYPpS1070 (where pS represents phosphoserine) sequence of p110β and serine 1070 as the site of autophosphorylation (Table I). In order to verify this finding, a p110β mutant in which serine 1070 was changed to alanine was created, and autophosphorylation of this mutant was compared with the wild-type enzyme in the presence of Mn2+. Fig. 2C shows that no significant phosphate incorporation into the mutant p110βS1070A of PI3Kβ took place while this mutant was still catalytically active as a lipid kinase (see below). Hence, results obtained by mass spectrometry and mutagenic analysis demonstrate that serine 1070 represents in fact the main site of autophosphorylation in p110β.

Both phosphorylation of the p85 adaptor by p110α and autophosphorylation of p110β down-regulate the enzymes’ lipid kinase activities (14–16, 20). p110β, like p110δ, autophosphorylates a serine residue at the extreme C terminus, which may also affect the catalytic activity of PI3Kβ. In order to test this assumption, we measured lipid kinase activities of p110β variants in which serine 1070 was mutated. Since the lipid kinase activity of PI3Kβ can be synergistically stimulated by Gbγ complexes and a tyrosine-phosphorylated peptide derived from the platelet-derived growth factor receptor, we measured formation of PtdIns-3,4,5-P3 under basal conditions and after stimulation of PI3Kβ variants with either stimuli in the presence of Mg2+ (11). Wild-type p110β and the nonphosphorylating p110βS1070A mutant exhibited the same enzymatic activity under basal conditions and after stimulation with Gbγ, phosphotyrosyl peptide, or both stimuli (Fig. 2D). This finding indicates that serine 1070 is not essential for the catalytic activity of p110β. However, purified mutant PI3Kβ het-
erodimers are less stable than the wild-type enzyme (data not shown). In order to mimic the effects of p110β autophosphorylation, mutants of p110β containing the negatively charged aspartic and glutamic acid instead of serine 1070 were employed. These p110βS1070D/E mutants no longer autophosphorylated (data not shown). Moreover, as shown in Fig. 2D, the lipid kinase activity of either p110βS1070D/E mutant was reduced by 4–7-fold under basal conditions and following stimulation with Gβγ and phosphotyrosyl peptides. This implies a regulatory function of the p110β autophosphorylation.

Next we created a peptide corresponding to the C terminus of p110β (WMHAHTVRKDYSR) as a pseudosubstrate in order to test whether the protein kinase activity of the autophosphorylated p110β was still intact. Applying mass spectrometry, no phosphorylation of this peptide by wild-type PI3Kβ was observed (data not shown). Moreover, as indicated in Fig. 2E, increased amounts of the C-terminal peptide did not influence the autophosphorylation of p110β by competition. Therefore, we assume that protein phosphorylation by PI3Kβ requires highly specific protein-protein interactions, and due to the lack of appropriate substrates the effect of p110β autophosphorylation on the protein kinase activity of the enzyme remains unknown so far.

Regulation of p110β Autophosphorylation—The finding that autophosphorylation of p110β on serine 1070 results in down-regulation of the lipid kinase activity of PI3Kβ (see Fig. 2D) suggests a regulatory function of this autophosphorylation. Therefore, one may suppose that the protein kinase activity like the lipid kinase activity of PI3Kβ is controlled by cell surface receptors. In order to address this hypothesis, we compared both kinase activities after incubation of PI3Kβ with increasing concentrations of Gβγ complexes and the tyrosine-phosphorylated peptide. As indicated, Fig. 3, A and B, shows that the lipid kinase activity of PI3Kβ was stimulated in a concentration-dependent manner by Gβγ (EC_{50} = 20 nM) or phosphotyrosyl peptide (EC_{50} = 5 nM). In contrast, neither Gβγ nor phosphotyrosyl peptide stimulated p110β autophosphorylation (see Fig. 3, A and B). Moreover, a combination of both stimuli led to a remarkable synergistic activation of PI3Kβ lipid kinase activity (see Fig. 2B) (11). However, even under these conditions, autophosphorylation of p110β was not enhanced regardless of whether Mg^{2+}, Mn^{2+}, or mixtures thereof were present (data not shown).

These observations suggest a high level of basal p110β autophosphorylation. Nonetheless, we found that in the presence of Mn^{2+}, the stoichiometry of phosphorylation was maximally 0.5 mol of phosphate/mol of p110β (Fig. 3C), arguing against a high basal autophosphorylation as the reason for the missing regulation of PI3Kβ protein kinase activity in vitro. Moreover, no differences in the time course of p110β autophosphorylation occurred, regardless of whether Gβγ or tyrosine-phosphorylated peptide were present. It is interesting that autophosphorylation peaked after more than 30 min under in vitro conditions. Taken together, the presented data do not exclude the possibility that p110β autophosphorylation may be involved in receptor-independent regulation of PI3Kβ enzymatic activity.

Autophosphorylation of p110γ Is Stimulated by Gβγ—Major characteristics of PI3Kγ autophosphorylation such as Mn^{2+} dependence and inhibition of lipid kinase activity resemble those of class I_γ PI3Kζ and δ autophosphorylation. However, in contrast to class I_γ kinases, a significant autophosphorylation of p110γ occurs in the presence of Mg^{2+}. Furthermore, autophosphorylation does not change the lipid kinase activity of PI3Kγ (11, 28). These findings suggest a role for PI3Kγ autophosphorylation different from the class I_γ PI3Kζ isoforms. Our observation that Gβγ stimulates p110γ autophosphorylation further supports this assumption (11, 12). Since others have reported an inhibitory effect of Gβγ on PI3Kζ protein kinase activity (29), we reexamined Gβγ-induced p110γ autophosphorylation using recombinant purified protein (Fig. 4). In the absence of lipid vesicles, Gβγ did not increase autophosphorylation of p110γ. In contrast, the addition of lipid vesicles led to a significant Gβγ-dependent stimulation of p110γ autophosphorylation regardless of whether the lipid vesicles contained PtdIns-4,5-P_2 (see Fig. 4A). These data may indicate that the orientation of proteins on the lipid bilayer surface facilitates the interaction of Gβγ with PI3Kγ. Interestingly, we observed phosphorylation of p101, which could not be stimulated by Gβγ even in the presence of lipid vesicles. Since in these experiments, a GST-p101/p110γ heterodimer was analyzed (see Fig. 4B), we also used a His-p110γ/p101 heterodimer (Fig. 5A) in order to exclude the possibility that a bulky GST tag may influence the phosphorylation of PI3Kζ subunits. As indicated in the upper panel of Fig. 5B, phosphorylation of a p101 variant without a GST tag was not visible. Moreover, both Gβγ-stimulated p110γ autophosphorylation of the His-p110γ/p101 heterodimer (EC_{50} = 30 nM) and lipid kinase activity (EC_{50} = 10 nM) were comparable with the data obtained with the GST-p101/p110γ heterodimer (see Fig. 5B) (11, 12). Taken together, these results clearly demonstrate that, in contrast to class I_γ PI3Kζ, autophosphorylation of PI3Kγ is sensitive to Gβγ. Whereas under basal conditions autophosphorylation of p110γ increased linearly for a period of more than 90 min, the presence of Gβγ significantly accelerated this phosphorylation (Fig. 5C). In particular, autophosphorylation of p110γ reached its maximum after 20 min with a stoichiometry of about 0.95 mol of incorporated phosphate/mol of p110γ. The latter observation suggests the presence of one autophosphorylation site in p110γ.

Autophosphorylation of p110γ Does Not Inhibit PI3Kγ Lipid Kinase Activity—In order to examine the effect of p110γ autophosphorylation on PI3Kγ lipid kinase activity, p110γ was phosphorylated in the presence of Gβγ. The catalytic activity of this autophosphorylated PI3Kγ was compared with the non-phosphorylated counterpart using PtdIns-4,5-P_2 as the substrate (Fig. 6). No differences in the production of PtdIns-
indicated periods of time. At equilibrium, the stoichiometry of G-labeled PtdIns-3,4,5-P₃ and p110

Apparent molecular masses of marker proteins are indicated. Cells, subjected to SDS-PAGE, and analyzed by Coomassie staining.

Stimulated p110 (Autophosphorylated. Therefore, in contrast to the C-terminal
detailed under "Experimental Procedures." Representative autoradiographs are depicted at the top, whereas graph bars with mean values ± S.D. of three independent experiments are shown at the bottom. Lipid and protein kinase activities of PI3Kγ are illustrated as -fold stimulation of basal activities. C, autophosphorylation of p110γ is accelerated in the presence of Gβγ subunits. Purified His-p110γ/p101 was incubated with phospholipid vesicles and [γ-32P]ATP in the absence (open circles) or presence (closed circles) of 120 nM purified Gβγ, for the indicated periods of time. At equilibrium, the stoichiometry of Gβγ-stimulated p110γ autophosphorylation was ~0.95 mol of phosphate/mol of p110γ. The time course of one representative experiment out of three is shown.

3,4,5-P₃ were detected, regardless of whether PI3Kγ was autophosphorylated. Therefore, in contrast to the C-terminal autophosphorylation of p110β and p110δ, which both down-regulate lipid kinase activity of PI3K, autophosphorylation of p110γ has no obvious inhibitory effects on PI3Kγ enzymatic activity. Hence, we assumed that autophosphorylation of p110γ occurs at a site different from a serine residue at its C terminus.

Identification of the p110γ Autophosphorylation Site—Phosphoamino acid analysis revealed that p110γ autophosphorylates serine but not threonine or tyrosine residues (Fig. 7A). In order to identify the phosphorylated serine residue, in vitro [32P]Phosphate-labeled p110γ protein was cleaved using different proteases. The resulting peptides were separated by reversed-phase HPLC, and the radioactivity of each fraction was determined. After digestion with chymotrypsin, a phosphopeptide corresponding to the C terminus of p110γ was identified by MALDI-MS (m/z 1134.56) and nanoESI-MS (doubly charged ion with m/z 567.77), as shown in Fig. 7B. The site of modification within the C-terminal sequence 1093–1102 was determined by nanoESI-MS/MS (see Fig. 7B, lower panel). In particular, the C-terminal γ' fragment ion series and the loss of neutral H₃PO₄ (98 mass units) confirmed the sequence and identified phosphorylation of serine 1101. Thus, the MS data demonstrate that serine 1101 of p110γ is the site of autophosphorylation. To confirm this finding, a p110γ mutant in which serine 1101 was changed to alanine was examined for its autophosphorylation. As indicated in Fig. 7C, no significant Gβγ-stimulated phosphate incorporation into the p110γS1101A mutant took place. Hence, both class Iγ p110γ as well as class Iγ p110δ and p110ε isoforms autophosphorylate on a serine residue at the extreme C terminus.

Lipid Kinase Activity of Mutant PI3Kγ—Next we examined the in vitro lipid kinase activity of heterodimeric PI3Kγ variants containing either wild-type p110γ or a mutant p110γ in which serine 1101 was replaced by either alanine (see above) or the negatively charged aspartic and glutamic acid (Fig. 8A). No differences in the production of PtdIns-3,4,5-P₃ by these PI3Kγ variants were observed under both basal conditions and followed by stimulation with Gβγ. These data underline that autophosphorylation of p110γ does not inhibit PI3Kγ lipid kinase activity. Moreover, HEK293 cells were transiently transfected with wild-type or mutant PI3Kγ as well as with the G protein-coupled fMLP receptor, and Akt phosphorylation was subsequently determined. In the absence of PI3Kγ, no fMLP-induced phosphorylation of Akt was observed (data not shown), whereas in the presence of any PI3Kγ variants (i.e. the wild-type enzyme or the alanine, aspartate, or glutamate mutants), Akt phosphorylation was significantly stimulated by fMLP to a comparable extent (Fig. 8B). Hence, results obtained both in an in vitro assay using recombinant proteins and in a cell-based assay suggest that autophosphorylation of p110γ does not influence PI3Kγ lipid kinase activity and thus clearly differs from the autophosphorylation of class Iγ PI3K isoforms.

Mechanism of p110γ Autophosphorylation—In order to examine the mechanism of p110γ autophosphorylation, we used a peptide corresponding to the C terminus of p110γ (WFLHLV-LGIKQGEKHSA). However, this C-terminal peptide was not a substrate for PI3Kγ protein kinase activity, since a phosphorylation of this peptide by wild-type PI3Kγ was not detected using mass spectrometry (data not shown). Furthermore, the peptide neither influenced p110γ autophosphorylation under basal conditions nor in the presence of Gβγ (Fig. 9A). Moreover,
a kinase-defective p110γK833R mutant did not autophosphorylate, emphasizing that phosphorylation of purified PI3Kγ preparations was not due to the presence of a contaminant kinase activity copurifying with the lipid kinase (Fig. 9B). Last, co-incubation of p110γK833R with enzymatically active wild-type heterodimeric PI3Kδ did not result in phosphorylation of the mutant p110γK833R, whereas the wild-type enzyme autophosphorylated. Hence, a transphosphorylation mechanism can be excluded.

**DISCUSSION**

The present study describes the autophosphorylation sites of the G protein-sensitive class I PI3Kβ and -γ isoforms. The experimental approaches include mass spectrometric analysis of the posttranslationally modified proteins and site-directed mutagenesis, which are independent and complementary methods. With these strategies, we identified the C-terminal residues serine 1070 and serine 1101 of the catalytic subunits as the modified amino acids. Previously, the C-terminal serine 1039 was detected as the site of p110δ autophosphorylation (15). Hence, class PI3Kβ, -γ, and -δ isoforms share the extreme C terminus of the catalytic subunit as a common site of autophosphorylation, whereas p110α is not significantly modified, probably due to the lack of a serine residue in this region.
Recently, Williams and co-workers published the crystal structure of p110γ for a fragment comprising amino acid residues 144–1102 (31). Although serine 1101 was not resolved in this structure it is clear from the data that it should be just beyond the C-terminal helix k833, which lines the PtdIns-4,5-P₂ binding pocket. Therefore, from a sterical point of view, a preferential phosphorylation of this serine appears reasonable. Moreover, lipid and protein kinase activities may compete with each other (32, 33). This assumption is supported by recent data from Yart et al. (27) demonstrating that a “protein kinase-only” (PKO) mutant of p110β exhibited an even higher protein kinase activity than the wild-type enzyme. Our own studies did not indicate any difference in the autophosphorylation activity of p110γ, regardless of whether lipid substrates such as PtdIns-4,5-P₂ were present (data not shown). Other data are more complex. Bondeva et al. (22) reported that only those PKO p110γ variants showed an increased autophosphorylation activity that contained a class II or class III donor activation loop but not the class IV counterpart. In contrast, wild-type p110α and all PKO p110α variants phosphorylated p85 equally well, whereas PKO p110α variants containing a class II or class III donor activation loop exhibited autophosphorylation of the p110α subunit (20). The latter finding was unexpected, since p110α lacks a C-terminal serine. In this context, our observation of a residual phosphorylation of the p110αS1070A mutant may be of interest (see Fig. 2C). The fact that purified kinase-defective mutants of p110β were not detectably phosphorylated by a contaminating kinase activity rather indicates the presence of a second, quantitatively less important phosphorylation site in p110β, presumably at threonine 1063 (see Fig. 1A). Support for this assumption comes from MALDI-post-source decay data, which revealed a double phosphorylated C-terminal peptide; unfortunately, the signal was too weak for sequencing by nanoESI/MS-MS. A corresponding C-terminal threonine of p110α (see Fig. 1A) may be a candidate target for significant autophosphorylation by class II and III PKO p110α variants (20).

In contrast to the predominant autophosphorylation of p110β observed in this and previous studies (27), others have reported p85 phosphorylation as the major target of PI3Kγ autophosphorylation (25, 26). In order to explain the apparent discrepancies, one must consider the experimental conditions used in these studies. Roche et al. (25) added purified p85 to immunoprecipitated p110β and detected a phosphorylated p85 band, whereas the p110β band was not shown. More important, PI3Kγ autophosphorylation was examined with enzyme preparations immobilized to beads in those studies (25, 26). Interestingly, this may affect autophosphorylation, since we noticed an increased p85 phosphorylation when we examined PI3Kγ bound to Ni²⁺-nitrilotriacetic acid beads.² Hence, experimental conditions significantly influence in vitro autophosphorylation of PI3Kγ. Likewise, in addition to p110γ autophosphorylation (11, 12, 26), p101 phosphorylation by PI3Kγ has been reported (29). In fact, using a GST-p101 construct, we also observed in initial experiments a phosphorylation of p101, which, in con-

²C. Czupalla and B. Nürnberg, unpublished observations.
PI3Kβ and PI3Kγ Autophosphorylation

trast to p110γ autophosphorylation, was not stimulated by Gβγ. However, p110 phosphorylation was not detectable when a purified hexahistidine-tagged PI3Kγ heterodimer was used. Therefore, we cannot exclude the possibility that the artificial bulky GST tag may have facilitated phosphorylation of p110.

The data presented in this study as well as in other reports provide evidence that the C terminus of p110 is a common site of autophosphorylation for three out of four class I PI3K isoforms. Despite this conformity, PI3Kβ and -γ differ in all other biochemical characteristics of autophosphorylation. For instance, PI3Kγ, like the two other class I isoforms, PI3Kα and -δ, autophosphorylates preferentially in the presence of Mn2+ (see Fig. 1B and Refs. 14–16). In contrast, PI3Kγ exhibits a significantly stimulated autophosphorylation activity in the presence of Mg2+ as shown previously (11, 28) and in this study. Interestingly, in vitro most serine/threonine kinases are Mg2+-dependent, whereas many tyrosine kinases show a greater activity in the presence of Mn2+ (34). Conversely, for phosphorylase kinase, a metal ion-dependent dual kinase specificity was reported (35). The presence of Mg2+ causes serine phosphorylation of phosphorylase b, and Mn2+ activates threonine phosphorylation of angiotensin II (35). The basis for these properties are still unclear. In this context, it should be remembered that autophosphorylation, per se, is not a good indicator of protein kinase activity, since many ATP-binding proteins that are not protein kinases are known to autophosphorylate in vitro (32).

We also addressed the control of autophosphorylation by upstream regulators. Under basal conditions, PI3Kγ slowly autophosphorylated (i.e. 0.1 mol of phosphate was incorporated into 1 mol of p110 within 30 min). The addition of Gβγ accelerated phosphate incorporation by 8–10-fold, resulting in an almost stoichiometric phosphorylation (see Fig. 5, B and C). Interestingly, this effect was only seen in the presence of lipid vesicles, which is in contrast to results obtained by Bondev and co-workers (29). Since the EC50 values for the stimulation of lipid and protein kinase activities of PI3Kγ were concordant, we hypothesized that the molecular mechanisms of stimulation of these kinase activities are similar. In contrast, neither Gβγ nor phosphotyrosyl peptide stimulated autophosphorylation of PI3Kβ (see Fig. 3) even under low basal autophosphorylation conditions (i.e. in the presence of Mg2+). Unfortunately, in vitro data addressing a possible stimulation of autophosphorylation of PI3Kα and -δ by upstream regulators are missing. However, Vanhaesebroeck and co-workers (15) reported a CD28-mediated stimulation of C-terminal p110 phosphorylation under in vivo conditions.

Data obtained from aspartate and glutamate mutants of p110β suggest that a phosphorylated PI3Kβ displays a hampered lipid kinase activity (see Fig. 2D). Unfortunately, a more direct experimental approach (e.g. assaying the effect of autophosphorylation on lipid kinase activity) was inconclusive. In particular, we were unable to completely remove Mn2+, which is necessary for autophosphorylation but disturbed PI3Kβ lipid kinase activity, without the use of immobilizing agents before carrying out the lipid kinase assay. Nevertheless, our results with the p110β1070D/E mutants are consistent with data obtained from the other class Iα kinases. Autophosphorylation of PI3Kα and -δ and exchange of serine 1039 of p110β to aspartate or glutamate inhibited lipid kinase activity (14–16, 20). Possible explanations for this effect include an induction of structural/conformational changes of the phosphorylated enzyme or an impact on the phospho-transfer reaction or on the ATP/PtdIns-4,5-P2 interaction (15). Furthermore, based on the crystal structure of p110γ, Williams and associates (31) have speculated that a phosphorylated C terminus may be a sterical impediment for PtdIns-4,5-P2 substrate binding. Surprisingly, here we provide experimental evidence that autophosphorylation of the C terminus of p110γ does not inhibit lipid kinase activity as shown under in vitro conditions with a prephosphorylated wild-type enzyme and p110γ1101D/E mutants (see Figs. 6 and 8A). These mutants showed full activity on cellular effectors in HEK293 cells in vivo (see Fig. 8B). Hence, we assume that autophosphorylation of p110γ, which is primarily regulated by Gβγ, has functions distinct from regulating its lipid kinase activity. One possibility may be the existence of PI3Kγ binding partners that specifically interact with the autophosphorylated form of PI3Kγ. In fact, recent evidence suggests that PI3Kγ interacts not only with its principal regulators (i.e. Gβγ and Ras) but also with additional components of signaling cascades such as the β-adrenergic receptor kinase 1 (36).

We found that PI3Kβ and -γ did not phosphorylate peptides derived from their respective C terminus, and vice versa the peptides did not affect the autophosphorylation capacity of the enzyme (see Figs. 2E and 9A). Similar observations were reported for PI3Kα and -δ (15), whereas Beeton et al. (26) described that PI3Kα and -δ phosphorylated a p85-derived peptide containing serine 608. Mechanistically, PI3Kγ did not transphosphorylate (see Fig. 9B), which may indicate a high degree of substrate specificity of the protein kinase activity. Notably, auto- but not transphosphorylation has also been reported for the p110γ monomer and a phosphatidylinositol 4-kinase β (37, 38). Surprisingly, while we were searching for in vivo substrates of PI3K protein kinase activity, we failed to detect p110γ autophosphorylation in HL-60 and SF9 cells so far, which emphasizes the need for further investigations into the regulation, activity, and targets of PI3Ks in vivo.

Acknowledgments—We thank H. Lerch and J. Malkewitz for excellent technical assistance. We thank Drs. Bart Vanhaesebroeck and Michael Waterfield for providing baculoviruses and Dr. Reinhard Wetzker for the monoclonal anti-p110 antibody. We also thank Drs. Michael Beyermann and Andreas Steinmeyer for providing peptides. Valuable discussions with Drs. Reinhard Wetzker, Len Stephens, Phil Hawkins, Lewis Cantley, and Roland Piekorz are appreciated.

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**PI3Kβ and PI3Kγ Autophosphorylation**