Regulation of Phosducin-like Protein by Casein Kinase 2 and N-terminal Splicing*

Received for publication, June 26, 2002, and in revised form, November 11, 2002
Published, JBC Papers in Press, December 3, 2002, DOI 10.1074/jbc.M206347200

Jan Humrich, Christina Bernet, Tobias Grübel, Ursula Quitterer, and Martin J. Lohse‡

From the Institute of Pharmacology and Toxicology, University of Würzburg, Versbacher Strasse 9, 97078 Würzburg, Germany

Phosducin-like protein (PhLP) is a member of the phosducin family of G-protein βγ-regulators and exists in two splice variants. The long isoform PhLP L and the short isoform PhLP s differ by the presence or absence of an 83-amino acid N terminus. In isolated biochemical assay systems, PhLP L is the more potent Gβγ-inhibitor, whereas the functional role of PhLP s is still unclear. We now report that in intact HEK 293 cells, PhLPS inhibited Gβγ-induced inositol phosphate generation with ~20-fold greater potency than PhLP L. Radiolabeling of transfected HEK 293 cells with 32P revealed that PhLP s is constitutively phosphorylated, whereas PhLPS is not. Because PhLP L has several consensus sites for the constitutively active kinase casein kinase 2 (CK2) in its N terminus, we tested the phosphorylation of the recombinant proteins by either HEK cell cytosol in the presence or absence of kinase inhibitors or by purified CK2. PhLP s was a good CK2 substrate, whereas PhLPS and phosducin were not. Progressive truncation and serine/threonine to alanine mutations of the PhLP L N terminus identified a serine/threonine cluster (Ser-18/Thr-19/Ser-20) within a small N-terminal region of PhLP L (amino acids 5–28) as the site in which PhLP L is phosphorylated. CK2 phosphorylation of phosducin reduces the affinity for Gβγ, and as a consequence, phosducin losess its ability to regulate G-protein function. In the case of PhLP, phosphorylation as a regulatory mechanism has been suggested but has never been demonstrated (10). Here we report that the Gβγ-regulatory function of PhLP s is inhibited in cells by N-terminal phosphorylation via casein kinase 2 (CK2), whereas PhLP L lacks such a regulatory mechanism. CK2 is a constitutively active and ubiquitously expressed serine/threonine kinase (12, 13). Our findings further provide strong evidence that the extended N terminus of PhLP L becomes autoinhibitory upon phosphorylation by CK2. In contrast, alternative splicing leads to PhLP s, which is not a substrate for CK2 and thus escapes this inhibitory mechanism.

EXPERIMENTAL PROCEDURES

Materials—[32P]ATP, [32P]orthophosphoric acid, and myo-[2-3H]-inositol were purchased from PerkinElmer Life Sciences. The kinase inhibitors staurosporine, BAPTA-AM, and 5,6-dichloro-1-β-glucuronobenzimidazole (DRB) were purchased from Calbiochem. Heparin sodium salt (average molecular weight 6,000) was from Sigma. Primary antibodies used were rabbit polyclonal anti-PhLP-CT. This synthetic peptide (CHSEDSDLIED) was from Sigma. Protein A-Sepharose was obtained from Amersham Biosciences. Phosphorimaging and quantification were done on a FLA-3000 from Fuji. ScanProsite was accessed at www.expasy.ch.

Construction of Expression Vectors—All of the cDNAs used in these studies were subcloned into pcDNA3. The cDNA for PhLP L was originally cloned from rat brain (2). The construction of deletion mutants and Ser/Thr to Ala mutants of PhLP L were done by a PCR-based strategy and confirmed by automated sequencing.

The PhLP mRNA exists in two major splice variants coding for two proteins, which differ by the presence (PhLP L) or absence (PhLP s) of the N-terminal 83 amino acids (1, 4). Subsequent studies on diverse tissues demonstrated that PhLP s is expressed in many organs at high protein levels (e.g. brain, heart, and liver), whereas PhLP L protein levels could not or only hardly be detected in these organs (4, 5). Direct comparison of the affinities toward Gβγ showed a 15-fold weaker interaction for PhLP s compared with phosducin or PhLP L (5). Therefore, PhLP L has been believed to play only a minor role in G-protein regulation. Recently, it was reported that PhLP L could be purified from cultured bovine chromaffin cells where it might inhibit nicotine-stimulated exocytosis of catecholamines by a pathway involving a Gβγ-ADP-ribosylation factor 6 complex (6). These findings suggested that alternative splicing of PhLP L might indeed occur in at least one organ or under specific conditions and therefore might play a role in differential functions of the PhLP isoforms.

Regulation of the homologous protein phosducin has been found to occur by phosphorylation via the serine/threonine kinases PKA, GRK, or CaM-dependent kinase II (7–11). Phosphorylation of phosducin reduces the affinity for Gβγ, and as a consequence, phosducin losess its ability to regulate G-protein function. In the case of PhLP, phosphorylation as a regulatory mechanism has been suggested but has never been demonstrated (10). Here we report that the Gβγ-regulatory function of PhLP L is inhibited in cells by N-terminal phosphorylation via casein kinase 2 (CK2), whereas PhLP s lacks such a regulatory mechanism. CK2 is a constitutively active and ubiquitously expressed serine/threonine kinase (12, 13). Our findings further provide strong evidence that the extended N terminus of PhLP L becomes autoinhibitory upon phosphorylation by CK2. In contrast, alternative splicing leads to PhLP s, which is not a substrate for CK2 and thus escapes this inhibitory mechanism.
Regulation of PhLP in Living Cells

Cell Culture, Transient Transfection, and Determination of Inositol Phosphates—Human embryonic kidney (HEK) 293 cells were grown in DMEM, 10% fetal calf serum and were kept in 7% CO2 humidified atmosphere. Cells were transfected by using the CaPO4 method (16) on 10-cm dishes at 70% confluency with a constant total amount of DNA. Transfection efficiency was usually between 60 and 80% and was equal for all of the cDNAs used as controlled by transfection of different green fluorescent protein-tagged constructs. For determination of inositol phosphates, cells were seeded in six-well plates and labeled with myo-[2-3H]inositol (2 µCi/ml; specific activity 21 Ci/mmol) for 16 h in isothiol-free RPMI 1640 medium containing 0.2% fetal calf serum. Unlabelled cells from the same transfection also seeded in six-well plates were used for Western blotting to control expression levels. After labeling, cells were washed once in incubation buffer (138 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.6 mM CaCl2, 1 g/liter glucose, 20 mM Na+-HEPES, pH 7.3) and then incubated at 37 °C for 20 min in incubation buffer containing 10 mM LiCl. Reactions were stopped, and inositol phosphates were extracted as described previously (17). Inositol determinations were performed in triplicates, and results were analyzed as the means ± S.E. of at least three independent experiments. ANOVA and post-test comparison (Bonferroni) were performed as appropriate. For the control of expression levels, cells of one well were lysed in 250 µl of hypertonic lysis buffer (1% Triton X-100, 20 mM Tris base, pH 10.5) for 15 min on ice and then centrifuged. Supernatants were analyzed by Western blots using polyvinylidine difluoride membranes (Millipore) and anti-PhLP-CT antibodies. Detection was performed with goat anti-rabbit-horseradish peroxidase antibodies and the Uptilight horseradish peroxidase-blocking kit (Interchim).

Phosphorylation of Phosducins—Phosducin, PhLP5, and PhLP6 were purified from Escherichia coli C-terminally His6-tagged proteins (5). PKA phosphorylation was performed as described (10), and phospho-proteins were visualized by exposure to Biomax MS Films (Eastman Kodak Co.). CK2 kinase assays were performed with recombinant human CK2 enzyme (Roche Molecular Biochemicals) or cell extracts. In kinase assays using recombinant human CK2, a 50-µl volume containing 400 nm recombinant PhLP5, PhLP6, or phosducin, CK2 buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl2, 5 mM dithiothreitol), 0.04 pmol of CK2, and 200 µM ATP (1 µCi of [32P]ATP) was incubated at 30 °C for 30 min or at 37 °C for 120 min. The reaction was terminated by boiling samples in Laemmli buffer followed by SDS-PAGE, Coomassie Blue staining, and phosphorimaging. For kinase assays using HEK cell cytosol, a lysate was prepared by resuspending cells of one 15-cm dish in 5 ml of CK2 buffer (supplemented with 1 mM PMSF), disrupting the cells by sonication and clearing the lysate by centrifugation (20,000 × g, 10 min). Protein content was determined in the supernatant by the Bradford method. Phosphorylation was then performed by adding 150 µg of total protein of cell extract to a 250-µl reaction with 400 nm His6-tagged protein and 10 µCi of [32P]ATP and incubated at 30 °C for 30 min. Reactions were then placed on ice for 5–15 min to quench phosphorylation. After centrifugation, the supernatants were washed with ice-cold pull-down buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl), supplemented with 50 µl of Ni-NTA-agarose (Qiagen), and rotated at 4 °C for 10 min. The beads were then washed, boiled, and analyzed by SDS-PAGE and phosphorimaging. Phosphorylation of PhLP in intact HEK 293 cells was done essentially as described with minor modifications (18). 48 h after transfection and 36 h after seeding in six-well plates, HEK 293 cells were washed in phosphate- and serum-free DMEM supplemented with 20 mM Na+-HEPES, pH 7.4, (labeling buffer) and then incubated in the same buffer for 30 min. Labeling of the intracellular ATP pool was then performed with 200 µCi/ml [32P]orthophosphoric acid (specific activity 10 mCi/mM) for 2 h at 30 °C. Where indicated, cells were stimulated with 1 mM carbachol for 5 min and then washed immediately on ice and washed once with ice-cold buffer. The cells were then lysed on ice with 1 ml of lysis buffer (300 mM NaCl, 50 mM NaF, 5 mM NaPO4, 5 mM EDTA-Na2, 0.1 mM Na3VO4, 1% Triton X-100, 0.01% NaN3, 50 mM Tris-HCl, pH 7.2, freshly supplemented with 10 mM iodoacetamide and 1 mM PMSF). After 20 min, the Triton X-100-insoluble fractions were removed by centrifugation (10,000 × g for 15 min) and PhLP was immunoprecipitated with the supernatants by the anti-PhLP-α antibody precooled to protein A-Sepharose for 2 h at 4 °C. After washing, immunoprecipitates were subjected to SDS-PAGE, and radiolabeled PhLP was visualized and quantified by phosphorimaging. Expression of PhLP was controlled by Western blotting of an aliquot obtained from the centrifuged lysates (with the anti-PhLP-CT antibody).

Tissue Preparation and Dephosphorylation—Mouse heart, brain, adrenal gland, and embryo (day 10.5 post-conception) were homogenized in five volumes of the same lysis buffer as described above (containing phosphatase inhibitors). After centrifugation and adjustment of total protein levels in supernatants as indicated, samples were subjected to SDS-PAGE and Western blotting. In the case of dephosphorylation experiments, lysis was performed in 40% Tris base, pH 10.5, 1% Triton X-100, and 1 mM PMSF (phosphatase inhibitors were omitted), samples were cleared by centrifugation (20,000 × g for 10 min), and protein content was determined by the Bradford method. Dephosphorylation was then performed by adding 50 µg of HEK cell lysate or 100 µg of brain lysate to a 50-µl reaction with phosphatase buffer (50 mM Tris-HCl, pH 7.5, 2 mM MnCl2, 5 mM dithiothreitol, 0.1% Brj 35) and with or without 1000 units of lambda-phosphatase (8) (New England Biolabs) for 16 h at 37 °C. The reaction was terminated by boiling in Laemmli buffer followed by SDS-PAGE and Western blotting.

RESULTS

Inhibition of Inositol Phosphate Signaling in Intact HEK 293 Cells—To investigate the functional role of PhLP5 and PhLP6 as Gβγ-regulators in living cells, we subcloned the appropriate cDNAs into the pcDNA3 expression vector and determined the inositol phosphate formation stimulated by the transient co-transfection of phospholipase Cα2 (PLCα2) and G-protein subunits Gβγ as described previously (19, 20). Without addition of any Gβγ-inhibiting proteins, the transfection of PLCβ2 and Gα12 cDNA (3 µg each) enhanced basal inositol phosphate accumulation ~5-fold compared with mock-transfected cells (data not shown). Co-transfection of the cDNA encoding PhLP5 inhibited the Gβγ-stimulated production of inositol phosphates significantly (Fig. 1A). Unexpectedly, the short splice variant PhLP5C turned out to be more effective than the long splice variant PhLP5, although PhLP6 interacts with purified Gγ12 less efficiently than PhLP5 (5). Co-transfection of 8 µg of cDNA for each Gβγ-binding protein inhibited the total inositol phosphate formation by 32.2 ± 8.4% (n = 13) for

![Fig. 1. Inhibition of Gβγ-stimulated inositol phosphate generation in HEK 293 cells.](image-url)
PhLP, the IC₅₀ of PhLPL was 20-fold higher than that of PhLPS (13.2 and 0.64 μg cDNA/10-cm dish, respectively). B and C, Western blots demonstrating increasing protein levels of PhLPL (B) and PhLPS (C) under the same conditions as in panel A. Note that different exposure times of the films were used to cover the full range of protein expression. The polyclonal PhLP-CT antibody was used as described under “Experimental Procedures.”

PhLPₕ, 78.3 ± 3.8% (n = 7) for PhLPₕ, and 75.0 ± 4.0% (n = 3) for GRK2-K220R, which served as positive control (21). Because inositol phosphate levels of untransfected control cells were ~20% of the inositol phosphate levels of PLCβ₂, Gßγ₂ co-expressing cells (data not shown), inhibition by ~80%, can be considered full inhibition. Thus, inhibition by PhLP was almost complete. The differences between control and PhLPLₕ and between PhLPLₕ and PhLPₕ were highly significant (p < 0.01). The different effects of PhLPL and PhLPS were not because of different protein expression as shown by Western blot analysis of the transfected cells (Fig. 1B). The blot with samples from three independent experiments shows equal expression of PhLPL and PhLPS within individual experiments and between different experiments.

To analyze further the different effects of PhLPLₕ and PhLPS on Gβγ-function, dose-response experiments were performed. The cDNA of PhLPLₕ or PhLPS was diluted with empty vector to maintain the total amount of transfected cDNA, and inositol phosphates were determined as before. As shown in Fig. 2A, the IC₅₀ of PhLPLₕ was 20-fold higher than that of PhLPS (13.2 and 0.64 μg cDNA/10-cm dish, respectively). Fig. 2, B and C, shows that the transfection of HEK 293 cells with increasing amounts of cDNA did indeed result in the expression of corresponding amounts of PhLPL or PhLPS, respectively.

**Fig. 2.** Concentration-dependent effects of PhLPLₕ and PhLPS on inositol phosphate generation. A, HEK 293 cells were transiently transfected with various amounts of cDNA for PhLPLₕ or PhLPS. The IC₅₀ of PhLPLₕ was 20-fold higher than that of PhLPS (13.2 and 0.64 μg cDNA/10-cm dish, respectively). B and C, Western blots demonstrating increasing protein levels of PhLPL (B) and PhLPS (C) under the same conditions as in panel A. Note that different exposure times of the films were used to cover the full range of protein expression. The polyclonal PhLP-CT antibody was used as described under “Experimental Procedures.”

**Phosphorylation of PhLPLₕ and PhLPS in HEK 293 Cells—**To analyze the role of phosphorylation in intact cells as a possible regulatory mechanism, we labeled HEK 293 cells with [³²P]orthophosphate and looked for basal- and receptor-stimulated phosphorylation. PhLP cDNAs were co-transfected with M₃ muscarinic receptor cDNA. The M₃ muscarinic receptor was chosen because of the prominent inositol phosphate signal that can be achieved by its stimulation (data not shown). 48 h after transfection, the receptors were stimulated with carbachol for 5 min (Fig. 3). PhLPLₕ exhibited a markedly higher degree of basal phosphorylation compared with PhLPS. However, in both cases, stimulation of the M₃ receptor by carbachol did not enhance the extent of phosphorylation (Fig. 3A, left panel). Fig. 3B summarizes a series of similar experiments demonstrating that the basal phosphorylation of PhLPLₕ was ~8-fold higher than that of PhLPS, but that carbachol did not significantly stimulate the phosphorylation of PhLPLₕ or of PhLPS. Expression was controlled by Western blotting with the PhLP-CT antibody and showed that both proteins were equally expressed (Fig. 3A, right panel). Overexpression of PhLPₕ did not affect
the phosphorylation state of PhLP$_L$. (data not shown). These findings demonstrate that PhLP$_S$ was constitutively phosphorylated in HEK 293 cells, whereas PhLP$_P$ only exhibited very low levels of phosphorylation.

Identification of CK2 as the Kinase Responsible for PhLP$_L$ Phosphorylation in HEK 293 Cells—To identify the responsible kinase for PhLP$_L$ phosphorylation, a computer analysis (ScanProsite) of the sequence of the N-terminal 83 amino acids of PhLP$_L$ revealed seven putative CK2 phosphorylation sites each within a classical CK2 phosphorylation motif ((S/T)-X-X-(D/E)) (see (Fig. 5A)). To test whether PhLP$_L$ could be a substrate of CK2 phosphorylation in vitro, we performed kinase assays with human recombinant CK2 and found that recombinant PhLP$_L$ was indeed a substrate, whereas PhLP$_S$ and phosducin were not (Fig. 4A). We used the potent but unspecific kinase inhibitor staurosporine (22) and the intracellular Ca$^{2+}$ chelator BAPTA-AM (23, 24) to test their effects on recombinant CK2 and found no effect on PhLP$_P$ phosphorylation. On the other hand, we tested two substances that were known to inhibit CK2: the glycosaminoglycan heparin (EC$_{50}$ 3 nM) (12, 25) and the nucleoside derivative DRB (EC$_{50}$ 7 μM) (26, 27). In the in vitro kinase assay, 3 μM heparin completely abolished PhLP$_L$ phosphorylation by CK2 and 100 μM DRB markedly inhibited PhLP$_L$ phosphorylation. To determine whether CK2 is the predominant PhLP$_L$ kinase in HEK 293 cells, we performed kinase assays using HEK cell lysate and recombinant PhLP$_L$ with and without the addition of kinase inhibitors and recombinant PhLP$_S$ as well as phosducin (Fig. 4B). Previous studies have shown that CK2 is abundantly present in mammalian cell lysates (12, 28). Here we demonstrate that PhLP$_L$ but not PhLP$_S$ or phosducin was predominantly phosphorylated by HEK cell lysate. This phosphorylation was inhibited by heparin (3 μM) and DRB (100 μM), whereas staurosporine only weakly inhibited and BAPTA-AM weakly stimulated the phosphorylation of PhLP$_L$. These results provide strong evidence that CK2 is the predominant kinase in HEK 293 cells that constitutively phosphorylates PhLP$_L$. We then asked whether PhLP was also a substrate for PKA phosphorylation in vitro similar to PKA-dependent phosphorylation of the homologous protein phosducin. PKA kinase assays were done with the same concentration (400 nM) of purified recombinant phosducin, PhLP$_L$, and PhLP$_S$ (Fig. 4C). Phosducin was an excellent PKA substrate, whereas both PhLP variants were not. They roughly showed 10–20-fold less radioactivity incorporated than phosducin. These results demonstrate that the different members of the phosducin family exhibit differential regulation by different kinases.

Identification of a Small Regulatory Region in the PhLP$_L$ N Terminus—We then asked whether any particular region of the PhLP$_L$ N terminus was responsible for the weaker functional effects of PhLP$_L$ compared with PhLP$_S$ as well as for the constitutive phosphorylation in HEK 293 cells. As depicted in Fig. 5A, the N terminus of PhLP$_L$ contains several putative phosphorylation sites that occur in clusters of serines and threonines. Seven of these sites could serve as CK2 phosphorylation sites. We constructed PhLP$_L$ mutants by stepwise shortening of the N terminus. The five PhLP$_L$ constructs were as follows: L5 (amino acids 5–301 with the loss of Thr-2 and Thr-3), L29 (amino acids 29–301 with the additional loss of Ser-18, Thr-19, Ser-20 and Ser-25), L36 (amino acids 36–301), L46 (amino acids 46–301 with the additional loss of Ser-39, Ser-40, Ser-41, and Thr-42), and L58 (amino acids 58–301 with the additional loss of Ser-54 and Thr-57). The determination of Gβγ-dependent inositol phosphate generation showed that a region within amino acids 5–28 caused the only major step in the gain of inhibitory function from PhLP$_L$ to PhLP$_S$ as demonstrated by the functional difference between L5 and L29 (Fig. 5A, lower panel). The removal of the first 28 amino acids of PhLP$_L$ (as in L29, L36, L46, and L58) was sufficient to gain the full Gβγ-inhibitory activity of PhLP$_S$. Western blots showed that all of the constructs with the exception of L5 were stable and that the expression levels of the constructs were comparable (Fig. 5B).

Fig. 4. Phosphorylation of recombinant phosducin and PhLP by HEK cell lysate, recombinant casein kinase 2, and protein kinase A. Equimolar concentrations (400 nM) of recombinant C-terminally His$_8$-tagged PhLP$_L$ (14.04 μg/ml), PhLP$_S$ (10.3 μg/ml), and phosducin (11.62 μg/ml) purified from E. coli were phosphorylated by either recombinant human casein kinase 2 or by HEK cell lysate or purified catalytic subunit of PKA. A, recombinant human CK2 (0.04 milliunit/reaction) was used to phosphorylate recombinant PhLP$_L$, PhLP$_S$, and phosducin at 30 °C for 30 min. After SDS-PAGE and Coomassie Blue staining (Coomassie), the gel was subjected to phosphorimaging ($^{32}$P). CK2 phosphorylated PhLP$_L$ efficiently, whereas PhLP$_S$ was only phosphorylated weakly (21% PhLP$_L$) and phosducin was not (2% PhLP$_L$). The concentration of the inhibitors used was 1 μM for staurosporine (Stauro), 100 μM for BAPTA-AM (BAPTA), 3 μM for harrpin, and 100 μM for DRB. Me$_2$SO (DMSO) (1% v/v) served as solvent control. B, HEK 293 cell lysate extracted as detailed under “Experimental Procedures” was used to phosphorylate recombinant PhLP$_L$ in the absence or presence of kinase inhibitors as well as PhLP$_S$ and phosducin at 30 °C for 30 min. Recombinant proteins were pulled down by Ni-NTA-agarose and processed as described under “Experimental Procedures.” Control denotes HEK cell lysate without any recombinant protein and reflects background phosphorylation. Shown is the gel as visualized by phosphorimaging ($^{32}$P) and by Coomassie Blue staining (Coomassie). Ph LP$_L$ phosphorylation was inhibited by harrpin and DRB (both known to inhibit CK2) but not by staurosporine or BAPTA, whereas PhLP$_S$ was only weakly phosphorylated. C, recombinant phosducin, PhLP$_L$, and PhLP$_S$ were phosphorylated by the catalytic subunit of PKA (100 units) at 30 °C for 15 min. Reactions were stopped by Laemmli buffer, boiled, and separated on 12.5% SDS-PAGE. Shown is the film ($^{32}$P) and the corresponding gel (Coomassie) to demonstrate that PKA phosphorylation of PhLP isoforms is not significant compared with phosducin.
Fig. 5. Progressive truncation of the PhLP_L N terminus revealed a short regulatory region between amino acids 5 and 28. A, the extended N terminus of PhLP_L contains several clusters of putative phosphorylation sites (S, serine; T, threonine) as depicted in the upper part of the panel. Arrows indicate the possible CK2 phosphorylation sites, whereas the numbers over the arrowheads indicate the amino acids C-terminal of the cutting point of the truncated constructs of PhLP_L. The removal of the first 28 amino acids of PhLP_L (L29, L29) was sufficient to gain the same Gβγ-inhibitory effect as PhLP_s whereas removing the first four amino acids (L5) was not. Additional progressive truncation did not change this effect any further. The inhibition of the inositol phosphate signal was by 42 ± 11% (n = 5) for PhLP_s, 52 ± 9% (n = 5) for L5, 84 ± 1% (n = 3) for L29, 85 ± 2% (n = 3) for L36, 84 ± 1% (n = 3) for L46, 85 ± 2% (n = 5) for L58, and 84 ± 3% (n = 5) for PhLP_s (***, p < 0.01; ***, p < 0.001 versus PhLP_s). B, expression control of the five N-terminally truncated constructs in comparison to that of PhLP_s and PhLP_L. Transfections were done as in inositol phosphate measurements, and 20% of the lysate of one well of a 6-well plate/lane was used. Shown is one of two Western blots with similar results. Used was the PhLP-CT antibody as described under “Experimental Procedures.”

Effect of Mutation of Serine 18, Threonine 19, and Serine 20 to Alanines—We next asked whether the putative CK2 phosphorylation sites of the region compromising amino acids 5–28 might play a functional role in PhLP_s regulation, and if so, which of the four candidates (Ser-18, Thr-19, Ser-20, and Ser-25) was involved. Two mutants were constructed, PhLP_sA18–20 in which Ser-18, Thr-19, and Ser-20 were changed to alanines, and PhLP_sA25 in which Ser-25 was changed to an alanine. These constructs were tested for their ability to inhibit Gβγ-dependent inositol phosphate generation. PhLP_sA18–20 showed the same Gβγ-inhibitory capability as PhLP_s and L29 (Fig. 6A), whereas PhLP_sA25 exhibited the same functional capability as PhLP_sA25. The differences between the effects of PhLP_s and PhLP_sA25 on the one hand and PhLP_s, L29, and PhLP_sA18–20 on the other hand were highly significant (Fig. 6A). Again, equal expression of the constructs was demonstrated in Western blot experiments (Fig. 6B). These data suggest that the cluster of Ser-18, Thr-19, and Ser-20 contains the phosphorylation sites responsible for the diminished Gβγ-inhibitory effects of PhLP_s, whereas Ser-25 most probably was not involved. Overexpression of PhLP_s in a 4-fold excess over PhLP_s or PhLP_sA18–20 reduced the inhibition of the latter to the level exerted by PhLP_s alone (Fig. 6C), suggesting that PhLP_s competed with PhLP_s for inhibiting Gβγ. A 4-fold overexpression of PhLP_s over PhLP_s was controlled by Western blots (data not shown).

In Vivo Relevance of PhLP Splicing and Phosphorylation—Finally, we asked whether the observed phenomenon of PhLP regulation via N-terminal phosphorylation or splicing might play a role in different mouse organs, which had been described to contain considerable amounts of PhLP_s (heart and brain) (4,
and might also contain PhLP$_S$ (adrenal gland) (6). In addition, embryos were chosen because CK2 plays an essential role in developing cells and cell cycle progression (13). Therefore, we expected to detect a high level of phospho-PhLP$_L$. PhLP$_L$ was detected by Western blotting in all three organs as well as in day 10.5 embryo and in control (empty vector) transfected HEK 293 cells (Fig. 7A). In contrast, PhLP$_S$ was present in clearly detectable amounts only in the adrenal gland and in HEK 293 cells after transfection of the PhLP$_S$ cDNA (Fig. 7A). After longer exposure of the film, PhLP$_S$ seemed to be present also in heart and brain but not in HEK 293 cells or embryo (data not shown). In most instances, PhLP$_R$ ran as a doublet, one band corresponding to the position of recombinant PhLP$_R$ purified from E. coli (Fig. 7B). HEK 293 cells and mouse brain lysate were then treated with $\lambda$-PPase, which has been described to efficiently remove phosphates from serine, threonine, and tyrosine residues (29). This resulted in the loss of the PhLP$_R$, high molecular mass form as detected by Western blotting, whereas the low molecular mass form of PhLP$_R$ became detectable in HEK 293 cells and was enhanced in mouse brain (Fig. 7C). The contention that the upper band represents phosphorylated PhLP$_R$ was supported by the observation that phosphorylation of PhLP$_R$ by CK2 in vitro caused a reduction of gel mobility (Fig. 7D). Together, these data demonstrate that there is constitutive phosphorylation of PhLP$_R$ in different mouse tissues and that in brain and adrenal gland non-phosphorylated PhLP$_R$ seems to play a role.

**DISCUSSION**

G-protein function is regulated by different classes of proteins, most notably the RGS proteins and the members of the phosducin family. The members of the phosducin family bind to the $G_{\beta\gamma}$-subunits (30, 31) and therefore inhibit G-protein function. This might result in the disruption of the G-protein cycle, (32, 33) and in the inhibition of $G_{\beta\gamma}$-mediated effects like activation of PLC$\beta$ isoforms and G-protein-coupled receptor kinase 2 (9, 34, 35). In this work, we used the activation of a $G_{\beta\gamma}$-dependent phospholipase C$_{\beta2}$ in HEK 293 cells as a functional readout of $G_{\beta\gamma}$-inhibition to demonstrate that PhLP inhibits $G_{\beta\gamma}$-functions in living cells. According to present knowledge, PhLP exists in two isoforms: 1) the long variant PhLP$_L$ with an additional N terminus of 83 amino acids and 2) the short variant PhLP$_S$, which lacks this N terminus (Figs. 5A and 8 (1)). However, the functional differences of these two isotypes in living cells remained to be elucidated, because (a) in vitro experiments showed that the short form, which lacks one $G_{\beta\gamma}$-binding region according to structural data (Fig. 8) (31), exhibited a markedly reduced binding affinity toward $G_{\beta\gamma}$-subunits compared with PhLP$_L$ and phosducin (2, 5) and (b) tissue levels of the PhLP isoforms appropriate for a physiological role in $G_{\beta\gamma}$-inhibition could only be demonstrated for the long form, PhLP$_L$ (4, 5). This finding appeared to suggest that the physiological $G_{\beta\gamma}$-regulator should be the long form, PhLP$_L$. Our present data show that in contrast to this hypothesis, PhLP$_S$ is an effective regulator of $G_{\beta\gamma}$-function in HEK 293 cells. The yeast phosducin homolog resembles PhLP$_S$ in size and domain structure (Fig. 8), suggesting that important $G_{\beta\gamma}$-regulating properties are contained in these proteins. Furthermore, our data suggest that the different forms of PhLP cooperate in inhibiting $G_{\beta\gamma}$ to a variable extent in that the phosphorylated form of PhLP$_L$ appears to have a partial dominant negative effect on the inhibitory function of PhLP$_S$.

PhLP$_S$ was found in mouse adrenal gland in amounts comparable with the levels of PhLP$_L$ in mouse brain and heart. In
FIG. 8. **Topology model of phosducin, PhLP<sub>L</sub>, and PhLP<sub>S</sub> and the yeast phosducin homolog derived from the crystal structure of phosducin (31).** Shown are the phosducin structural domains. The N terminus consists of three α-helices with Gβγ-binding regions and large unresolved areas. The C terminus is a thioredoxin domain consisting of five β-sheet and three flanking α-helices (not marked) with Gβγ-binding regions. PhLP<sub>L</sub> has all five homologous Gβγ-contact regions, and PhLP<sub>S</sub> bears four of the five Gβγ-contact regions and resembles the yeast phosducin homolog, which also does not contain the first α-helix and first Gβγ-binding region. As in phosducin, the PhLP<sub>L</sub> N terminus is subject to regulation by phosphorylation (a), most probably by CK2, and this seems to play an important role in vivo. A further possibility for a more long-term activation of PhLP would be alternative splicing to the short variant PhLP<sub>S</sub> (b), which lacks the possibility of N-terminal regulation by phosphorylation and seems to be relevant in the adrenal gland. In primitive eukaryotic organisms such as yeast, the "short phosducin" might be a sufficient principle of Gβγ-regulation.

Taken together, these findings indicate a regulatory function of the N terminus of PhLP<sub>L</sub> and underline the role of N-terminal regulation of the members of the phosducin family. Our data also show that PhLP isoforms differ in the mode of activity control and that the Gβγ-inhibitory effects of PhLP<sub>L</sub> are subject to regulation via the N-terminal domain. Phosphorylation of the N terminus, in vivo most probably by CK2, diminishes the ability of PhLP<sub>L</sub> to inhibit Gβγ-functions. The analysis of PhLP expression in mouse organs shows that both mechanisms are used in vivo.

**REFERENCES**

1. Miles, M. F., Barhite, S., Sganga, M., and Elliott, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10831–10835

2. Schröder, S., and Lohse, M. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 2100–2104

3. Thibault, C., Sganga, M. W., and Miles, M. F. (1997) *J. Biol. Chem.* **272**, 12253–12256

4. Thibault, C., Feng Wang, J., Charnas, R., Mirel, D., Barhite, S., and Miles, M. F. (1999) *Biochim. Biophys. Acta* **1444**, 346–354

5. Schröder, S., and Lohse, M. J. (2000) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **362**, 435–439

6. Genoise, M., Vitale, N., Chasserot-Golaz, S., and Bader, M. F. (2000) *FEBS Lett.* **480**, 184–188

7. Lee, R. H., Brown, B. M., and Lolley, R. N. (1990) *J. Biol. Chem.* **265**, 15860–15866

8. Bauer, P. H., Müller, S., Puzicha, M., Pippig, S., Obermaier, B., Helmreich, E. J., and Lohse, M. J. (1992) *Nature* **358**, 73–76

9. Yoshida, T., Willardson, B. M., Wilkins, J. F., Jensen, G. J., Thornton, B. D., and Buitenhuys, M. W. (1994) *J. Biol. Chem.* **269**, 24505–24507

10. Ruiz-Gomez, A., Humrich, J., Murga, C., Quitterer, U., Lohse, M. J., and Mayor, F. Jr. (2000) *J. Biol. Chem.* **275**, 29724–29730

11. Thulin, C. D., Savage, J. R., McLaughlin, J. N., Truscott, S. M., Old, W. M., Ahn, N. G., Resing, K. A., Hamm, H. E., Eiten, M. W., and Willardson, B. M. (2001) *J. Biol. Chem.* **276**, 23865–23873

12. Allende, J. E., and Allende, C. C. (1995) *FASEB J.* **9**, 313–323

13. Pinna, L. A., and Meggio, F. (1997) *Mol. Endo.

14. Liu, F. T., Zinnecker, M., Hamaoka, T., and Katz, D. H. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 5322

15. Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T. M., Sutcliffe, J. G., and Lerner, R. A. (1982) *Cell* **28**, 477–487

16. Cullen, B. R. (1987) *Methods Enzymol.* **152**, 684–704

17. Quitterer, U., AbdAlla, S., Jarnagin, K., and Muller-Esterl, W. (1996) *Biochemistry* **35**, 13368–13377

18. Malecz, N., Bambino, T., Bencsik, M., and Nissenson, R. A. (1998) *Mol. Endo.}

---

a Asterisk in sequence denotes potential phosphorylation site.
Regulation of PhLP in Living Cells

crinol. 12, 1846–1856
19. Xu, J., Wu, D., Slepak, V. Z., and Simon, M. I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2086–2090
20. Quitterer, U., and Lohse, M. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10626–10631
21. Dicker, F., Quitterer, U., Winstel, R., Honold, K., and Lohse, M. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5476–5481
22. Ruegg, U. T., and Burgess, G. M. (1989) Trends Pharmacol. Sci. 10, 218–220
23. Billman, G. E. (1993) Am. J. Physiol. 265, H1529–H1535
24. Jiang, S., Chow, S. C., Nicotera, P., and Orrenius, S. (1994) Exp. Cell Res. 212, 84–92
25. Block, K., Boyer, T. G., and Yew, P. R. (2001) J. Biol. Chem. 276, 41049–41058
26. Zandomeni, R. O. (1989) Biochem. J. 262, 469–473
27. Kim, S. J., and Kahn, C. R. (1997) Biochem. Biophys. Res. Commun. 234, 681–685
28. Lin, A., Frost, J., Deng, T., Smeal, T., al-Alawi, N., Kikkawa, U., Hunter, T., Brenner, D., and Karin, M. (1992) Cell 70, 777–789
29. Zhuo, S., Clemens, J. C., Hakes, D. J., Barford, D., and Dixon, J. E. (1993) J. Biol. Chem. 268, 17754–17761
30. Lee, R. H., Lieberman, B. S., and Lolley, R. N. (1987) Biochemistry 26, 3983–3990
31. Gaudet, R., Bohm, A., and Sigler, P. B. (1996) Cell 87, 577–588
32. Muller, S., Straub, A., Schreiber, S., Bauer, P. H., and Lohse, M. J. (1996) J. Biol. Chem. 271, 11781–11786
33. Bauer, P. H., and Lohse, M. J. (1998) Naunyn-Schmiedeberg’s Arch. Pharmacol. 357, 371–377
34. Hekman, M., Bauer, P. H., Sobhleman, P., and Lohse, M. J. (1994) FEBS Lett. 343, 120–124
35. Schulz, K., Danner, S., Bauer, P., Schroder, S., and Lohse, M. J. (1996) J. Biol. Chem. 271, 22546–22551
36. Kim, Y. M., Barak, L. S., Caron, M. G., and Benovic, J. L. (2002) J. Biol. Chem. 277, 16837–16846