

Casein kinase II–mediated phosphorylation of lipin 1 β phosphatidate phosphatase at Ser-285 and Ser-287 regulates its interaction with 14-3-3 β protein

Received for publication, December 19, 2018, and in revised form, January 4, 2019. Published, Papers in Press, January 7, 2019. DOI 10.1074/jbc.RA118.007246

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Edited by F. Peter Guengerich

The mammalian lipin 1 phosphatidate phosphatase is a key regulatory enzyme in lipid metabolism. By catalyzing phosphatidate dephosphorylation, which produces diacylglycerol, the enzyme plays a major role in the synthesis of triacylglycerol and membrane phospholipids. The importance of lipin 1 to lipid metabolism is exemplified by cellular defects and lipid-based diseases associated with its loss or overexpression. Phosphorylation of lipin 1 governs whether it is associated with the cytoplasm apart from its substrate or with the endoplasmic reticulum membrane where its enzyme reaction occurs. Lipin 1 β is phosphorylated on multiple sites, but less than 10% of them are ascribed to a specific protein kinase. Here, we demonstrate that lipin 1 β is a *bona fide* substrate for casein kinase II (CKII), a protein kinase that is essential to viability and cell cycle progression. Phosphoamino acid analysis and phosphopeptide mapping revealed that lipin 1 β is phosphorylated by CKII on multiple serine and threonine residues, with the former being major sites. Mutational analysis of lipin 1 β and its peptides indicated that Ser-285 and Ser-287 are both phosphorylated by CKII. Substitutions of Ser-285 and Ser-287 with nonphosphorylatable alanine attenuated the interaction of lipin 1 β with 14-3-3 β protein, a regulatory hub that facilitates the cytoplasmic localization of phosphorylated lipin 1. These findings advance our understanding of how phosphorylation of lipin 1 β phosphatidate phosphatase regulates its interaction with 14-3-3 β protein and intracellular localization and uncover a mechanism by which CKII regulates cellular physiology.

PA³ phosphatase plays a central role in lipid metabolism (1–9) (Fig. 1). By catalyzing the dephosphorylation of PA to

produce diacylglycerol (10), the enzyme has a major impact on the synthesis of triacylglycerol and membrane phospholipids and on the abundance of the signaling molecule PA (1–9) (Fig. 1). Three genes encode PA phosphatase in humans (*i.e.* *LPIN1*, *LPIN2*, and *LPIN3*) and mice (*i.e.* *Lpin1*, *Lpin2*, and *Lpin3*) (11–13). Their protein products (*i.e.* lipin 1 (α , β , and γ isoforms), 2, and 3) share conserved domains at the N-terminal (NLIP) and C-terminal (CLIP) regions (11) (Fig. 2). The PA phosphatase activity depends on a conserved glycine within NLIP and the DXDX(T/V) catalytic motif in the haloacid dehalogenase-like domain within CLIP (14–16) (Fig. 2). Lipin 1 also has a transcriptional co-activator function that depends on the LXXIL motif in CLIP (17) (Fig. 2).

The importance of PA phosphatase to lipid metabolism is exemplified in humans and mice by cellular defects and lipid-based diseases associated with loss or overexpression of the enzyme activity. The loss of lipin 1 PA phosphatase activity causes rhabdomyolysis in humans and mice (18, 19), and the deficiency in mice is also characterized by hepatic steatosis during the neonatal period, lipodystrophy, insulin resistance, and peripheral neuropathy (11, 20). Polymorphisms in the human *LPIN1* gene are associated with insulin resistance and the metabolic syndrome (21), whereas the overexpression of the *Lpin1* gene in mice results in increased lipogenesis and obesity (22). Other disease conditions linked to lipin 1 PA phosphatase activity include inflammation and atherosclerosis (23, 24), liver disease (25–29), and cancer (30–34). Loss of human lipin 2 PA phosphatase activity causes chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anemia (Majeed syndrome) (35, 36), whereas genetic variations in the *LPIN2* gene are linked to type 2 diabetes (37). So far, no defects have been ascribed to the loss of lipin 3 PA phosphatase activity.

The mode of action and control of PA phosphatase activity is primarily regulated by its cellular location (3, 4, 38). Mouse lipin 1 is found in the cytoplasm, endoplasmic reticulum, and nucleus (4, 39, 40), and its cellular location is regulated by covalent modifications that include phosphorylation (41–43), sumoylation (44), acetylation (45), and interaction with 14-3-3 proteins (46). The phosphorylated form of lipin 1 is enriched in the cytoplasm, where it is physiologically inactive, whereas its dephosphorylated form is enriched in the membrane, where it catalyzes the PA phosphatase reaction (42, 43). The membrane

This work was supported, in whole or in part, by National Institutes of Health Grants GM050679 (to G. M. C.) and DK101946 (to T. E. H.) from the United States Public Health Service. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

¹ Supported by National Institutes of Health Training Grant T32 GM008715.

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³ The abbreviations used are: PA, phosphatidate; CKI and CKII, casein kinase I and II, respectively; PVDF, polyvinylidene difluoride; PMSF, phenylmethylsulfonyl fluoride; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

Phosphorylation of lipin 1β PA phosphatase by CKII

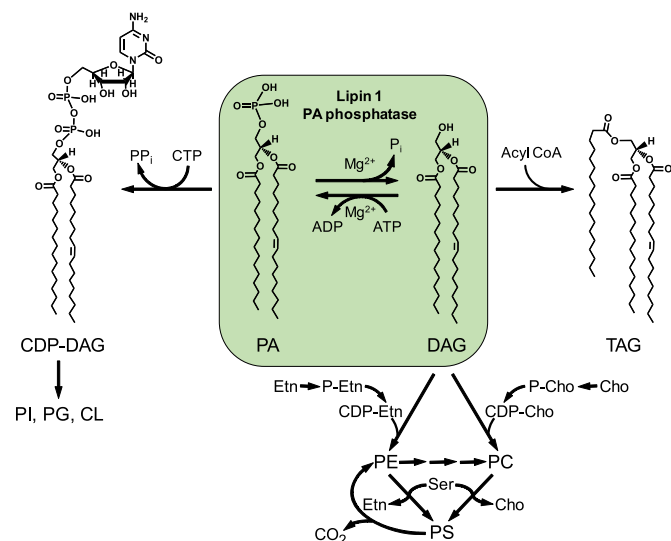


Figure 1. Central role of PA phosphatase in the synthesis of triacylglycerol and membrane phospholipids in mammalian cells. The structures of CDP-diacylglycerol, PA, diacylglycerol, and triacylglycerol are shown with the C16:0 and C18:1 fatty acyl groups. PA phosphatase plays a major role in governing whether cells utilize PA for the synthesis of triacylglycerol via diacylglycerol or for the synthesis of membrane phospholipids via CDP-diacylglycerol. The PA phosphatase reaction is counterbalanced by the conversion of diacylglycerol to PA. The major phospholipids phosphatidylcholine and phosphatidylethanolamine are synthesized from the PA-derived diacylglycerol via the CDP-choline and CDP-ethanolamine branches, respectively, of the Kennedy pathway. Phosphatidylcholine is also synthesized from phosphatidylethanolamine by the three-step methylation reactions using AdoMet as a methyl donor. Phosphatidylserine is derived from phosphatidylcholine or phosphatidylethanolamine via a base-exchange reaction, and its decarboxylation produces phosphatidylethanolamine. In addition to their roles in lipid synthesis, PA and diacylglycerol are known to facilitate membrane fission/fusion events (96–101) and play roles in vesicular trafficking (102–106). More comprehensive pathways of lipid synthesis may be found in Ref. 2. DAG, diacylglycerol; TAG, triacylglycerol; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Cho, choline; Etn, ethanolamine.

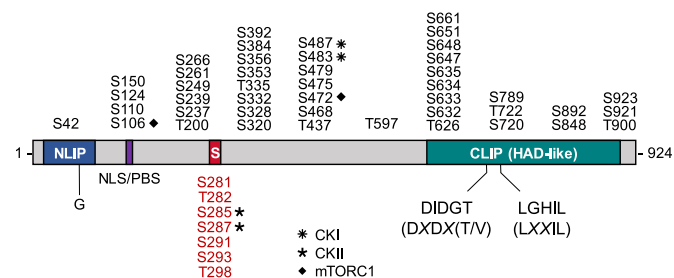


Figure 2. Domains/regions and phosphorylation sites of mouse lipin 1β. The diagram shows the conserved NLIIP (blue) and CLIP (haloacid dehalogenase (HAD)-like) (green) domains found at the N and C terminus, respectively; the conserved G in NLIIP and the catalytic (DXDXT) and transcriptional co-activator (LXXIL) motif sequences in CLIP; the nuclear localization signal/polybasic sequence (NLS/PBS, purple) region; and the serine-rich region (S, red). The serine (S) and threonine (T) residues known to be phosphorylated (42, 47–51) are grouped at their approximate regions in the protein, and those examined in this study are colored in red. The sites phosphorylated by CKI, CKII (this study), and mTORC1 are indicated.

association of the enzyme is facilitated by acetylation (45), whereas its association with the cytoplasm is facilitated by interaction with 14-3-3 proteins (46). The localization of lipin 1α to the nucleus, where it functions as a transcriptional coactivator (17), is facilitated by its sumoylation (44).

Phosphoproteomics and targeted phosphorylation studies have shown that mouse lipin 1β is phosphorylated on multiple

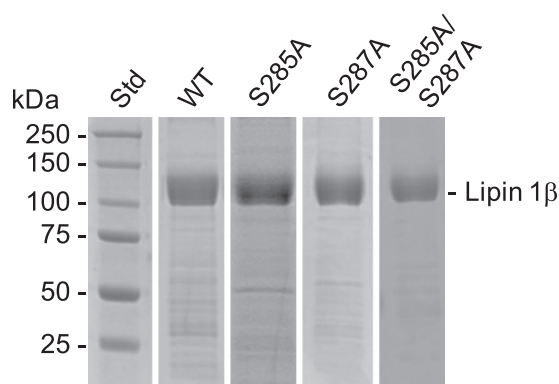


Figure 3. SDS-PAGE analysis of purified mouse lipin 1β proteins. Mouse FLAG-tagged lipin 1β was expressed in HeLa cells and purified by affinity chromatography. Samples of the purified preparations of the WT (3.9 μg), S285A (1.7 μg), S287A (4.8 μg), and S285A/S287A (1.4 μg) lipin 1β were subjected to SDS-PAGE and stained with Coomassie Blue. The positions of lipin 1β and molecular mass standards (Std) are indicated.

serine and threonine residues (42, 47–51) (Fig. 2). Except for mTORC1, which phosphorylates Ser-106 and Ser-472 (42, 47), and CKI, which phosphorylates Ser-483 and Ser-487 (52), the identities of the protein kinases that phosphorylate the remaining sites are unknown. Identifying protein kinases for lipin 1β phosphorylation is important to provide information on the signaling networks involved in its regulation under normal and disease states (53). In this work, we demonstrate that lipin 1β is phosphorylated by CKII, a conserved serine/threonine protein kinase that is essential for viability and cell cycle progression in mammalian cells (54–56). We show that CKII phosphorylates lipin 1β at Ser-285 and Ser-287. Moreover, the CKII phosphorylation of lipin 1β at Ser-285 and Ser-287 facilitates its interaction with 14-3-3β protein. This work advances the understanding of the phosphorylation-mediated regulation of lipin 1β PA phosphatase and demonstrates an unrecognized mechanism by which CKII regulates cell physiology.

Results

Lipin 1β is a substrate for CKII

Lipin 1β PA phosphatase is the predominant isoform in most tissues, and it is known to be phosphorylated on multiple residues (42, 47). The bioinformatics analysis of lipin 1β indicates that it is phosphorylated by a plethora of protein kinases (57). Of the putative kinases, CKII has a high probability of phosphorylating lipin 1β (57). Given this prediction, we examined whether lipin 1β is a target substrate for CKII. For this analysis, we used a purified preparation of mouse FLAG-tagged lipin 1β that was expressed in HeLa cells (Fig. 3) and examined its phosphorylation by following the incorporation of the radioactive phosphate from [γ - 32 P]ATP into the protein. The analysis of the reaction products by SDS-PAGE and phosphorimaging showed that lipin 1β is phosphorylated by CKII (Fig. 4A). By phosphoamino acid analysis, the 32 P-labeled lipin 1β was shown to contain phosphate on the residues of serine and threonine, with the former being a major site (76% of the total phosphorylation) (Fig. 4B). When lipin 1β was phosphorylated by CKII after pretreatment with λ-phosphatase (58) to remove phosphates from its endogenous phosphorylation, it did not show a difference in the extent of phosphorylation. This result

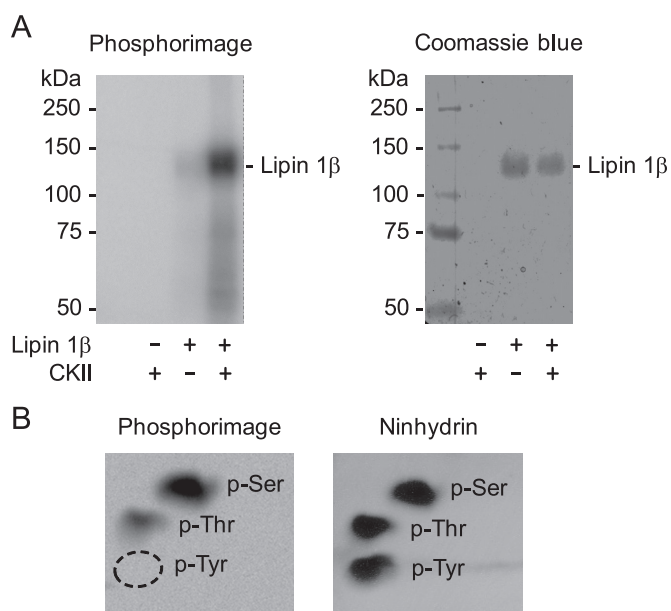


Figure 4. Lipin 1 β is phosphorylated by CKII on the serine and threonine residues. *A*, purified WT FLAG-tagged lipin 1 β (0.3 μ g) was incubated for 15 min at 30 °C in the presence (+) or absence (-) of 0.2 μ g of CKII and [γ - 32 P]ATP (2,000 cpm/pmol). The reaction mixtures were resolved by SDS-PAGE and subjected to phosphorimaging (*left*), followed by staining with Coomassie Blue (*right*). The positions of lipin 1 β and the molecular mass standards are indicated. *B*, 32 P-labeled WT lipin 1 β was incubated with 6 N HCl for 90 min at 110 °C. The acid hydrolysate was mixed with standard phosphoamino acids and resolved by two-dimensional electrophoresis on a cellulose TLC plate, which was subjected to phosphorimaging (*left*) and staining with ninhydrin (*right*). The positions of phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) are indicated. The experiments in *A* and *B* were repeated three times, and the data shown are representative.

indicates that the *in vitro* phosphorylation of lipin 1 β is not affected by its endogenous phosphorylation. We further characterized the lipin 1 β phosphorylation to confirm that it is a *bona fide* substrate for CKII. The protein kinase activity depended on the amount of CKII (Fig. 5*A*), the time of the reaction (Fig. 5*B*), the amount of lipin 1 β (Fig. 5*C*), and the concentration of ATP (Fig. 5*D*).

To determine whether the catalytic function of lipin 1 β is affected by its phosphorylation, the CKII-treated enzyme was measured for PA phosphatase activity by following the release of P_i from PA using the Triton X-100/PA-mixed micellar assay. Compared with the untreated control, which had a specific activity of 6 μ mol/min/mg, the CKII-phosphorylated lipin 1 β showed no significant difference in PA phosphatase activity.

CKII phosphorylates lipin 1 β in the serine-rich region

The phosphorylation of the serine-rich region of lipin 1 α facilitates its interaction with 14-3-3 proteins to promote a cytoplasmic localization (46). However, the protein kinase(s) involved in the phosphorylation is unknown. Analysis of the serine-rich region of lipin 1 β , which is identical to that of lipin 1 α , with the Phosphomotif Finder (59) and NetPhos (57) programs indicates that seven residues (*e.g.* Ser-281, Thr-282, Ser-285, Ser-287, Ser-291, Ser-293, and Thr-298) are putative sites for phosphorylation by CKII. To examine whether the residues are the phosphorylation sites of CKII, we utilized a 22-residue synthetic peptide whose sequence (RPSTPKSDSELVSK-SADRLTPK) was derived from the serine-rich region of lipin 1 β

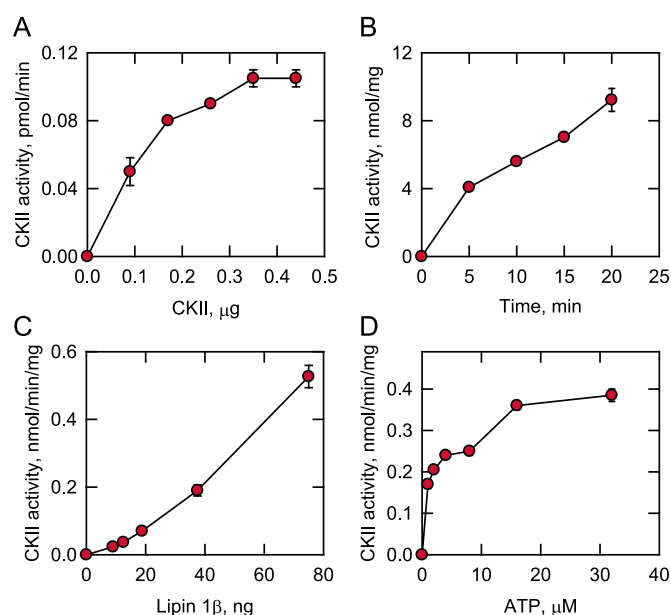


Figure 5. CKII activity on lipin 1 β depends on the reaction time and the amounts of CKII, lipin 1 β , and ATP. Lipin 1 β was incubated with CKII and [γ - 32 P]ATP at 30 °C, and its phosphorylation was monitored by following the incorporation of the radioactive phosphate into the protein. The reaction mixtures were spotted onto nitrocellulose papers, which were washed with 75 mM phosphoric acid and subjected to scintillation counting. The CKII reaction was conducted by varying the amount of CKII (*A*), the reaction time (*B*), the amount of lipin 1 β (*C*), and the concentration of ATP (*D*). *A*, *B*, and *C*, 50 μ M ATP; *A*, *C*, and *D*, 15 min; *A*, *B*, and *D*, 0.1 μ g of lipin 1 β ; *B*, *C*, and *D*, 0.2 μ g of CKII. The data shown are means \pm S.D. (error bars) from triplicate assays.

(residues 279–300). The phosphorylation of the lipin 1 β peptide was monitored by following the incorporation of the radioactive phosphate from [γ - 32 P]ATP into the peptide. The CKII activity on the lipin 1 β peptide depended on the amount of protein kinase (Fig. 6*A*) and the time of the reaction (Fig. 6*B*). The CKII activity was also examined with respect to the concentrations of the WT peptide (Fig. 6*C*) and ATP (Fig. 6*D*). In both cases, CKII activity followed Michaelis–Menten kinetics with apparent V_{max} and K_m values, respectively, for the WT peptide of 45 ± 5 nmol/min/mg and 146 ± 40 μ M and for ATP of 10.7 ± 0.8 nmol/min/mg and 6.2 ± 1.5 μ M. These results indicated that the lipin 1 β peptide (*i.e.* serine-rich region) is phosphorylated by CKII.

Mutational analysis of the lipin 1 β peptide identifies Ser-285 and Ser-287 as sites of phosphorylation by CKII

After determining the phosphorylation of the serine-rich region by CKII, we examined which of the seven serine/threonine residues is a target phosphorylation site. For this purpose, CKII activity was measured on the derivatives of the lipin 1 β peptide in which the alanine residue was substituted for the Ser or Thr residue (Fig. 7). A mutant peptide (referred to as 7A) in which all putative phosphorylation sites were changed to the alanine residues (*blue color*) served as a negative control for CKII activity. In seven peptides, one putative phosphorylation site was left intact (*red color*), and the remaining six putative sites were changed to the alanine residues (*blue color*). Of these peptides, the S285 and S287 peptides were phosphorylated by CKII at 71 ± 6.5 and $30 \pm 1.2\%$, respectively, of the WT peptide phosphorylation (Fig. 7). In contrast, the other mutant peptides

Phosphorylation of lipin 1 β PA phosphatase by CKII

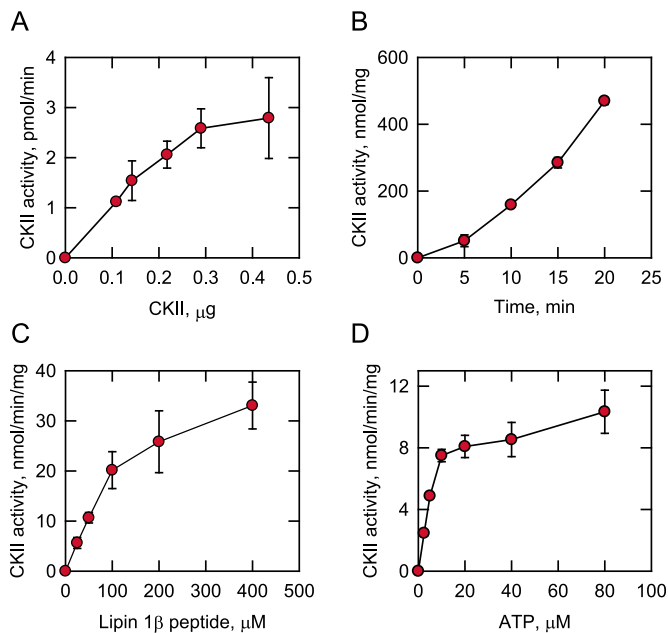


Figure 6. CKII activity on the lipin 1 β peptide depends on the reaction time and the amount of CKII, the lipin 1 β peptide, and ATP. The lipin 1 β peptide (RPSTPKSDSELVSKSADRLTPK) was incubated with CKII and [γ - 32 P]ATP at 30 $^{\circ}$ C, and its phosphorylation was monitored by following the incorporation of the radioactive phosphate into the peptide. The reaction mixtures were spotted onto P81 phosphocellulose papers, which were washed with 75 mM phosphoric acid and subjected to scintillation counting. The CKII reaction was conducted by varying the amount of CKII (A), the reaction time (B), the concentration of the lipin 1 β peptide (C), and the concentration of ATP (D). A, B, and C, 100 μ M ATP; A, C, and D, 15 min; A, B, and D, 100 μ M lipin 1 β peptide; B, C, and D, 0.2 μ g of CKII. The data shown are means \pm S.D. (error bars) from triplicate assays.

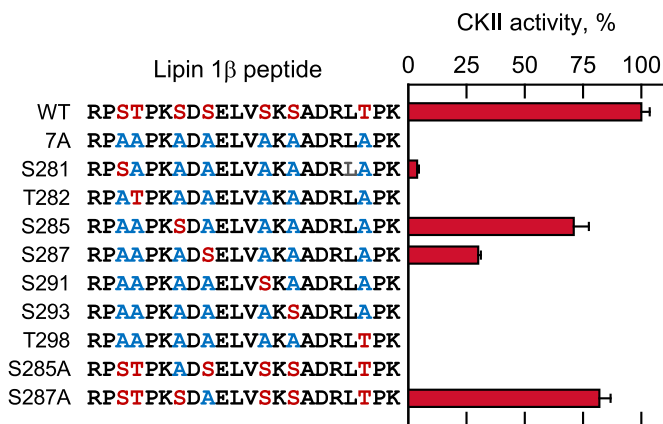


Figure 7. Analysis of lipin 1 β peptides by CKII identifies Ser-285 and Ser-287 as target phosphorylation sites. The indicated WT and mutant peptides (100 μ M) were incubated with 0.2 μ g of CKII and 100 μ M [γ - 32 P]ATP for 15 min at 30 $^{\circ}$ C, and the peptide phosphorylation was measured by following the incorporation of the radioactive phosphate into the peptide. The reaction mixtures were spotted onto P81 phosphocellulose papers, which were washed with 75 mM phosphoric acid and then subjected to scintillation counting. The phosphorylation levels of mutant peptides were normalized to that of the WT peptide. The data shown are means \pm S.D. (error bars) from triplicate assays.

were phosphorylated at the level of 4% or less (Fig. 7). These results indicate that Ser-285 and Ser-287 of the serine-rich region are the phosphorylation sites of CKII.

The phosphorylation of Ser-285 and Ser-287 was further examined by mutating the serine residues individually to alanine (*i.e.* S285A and S287A, blue color) without altering the

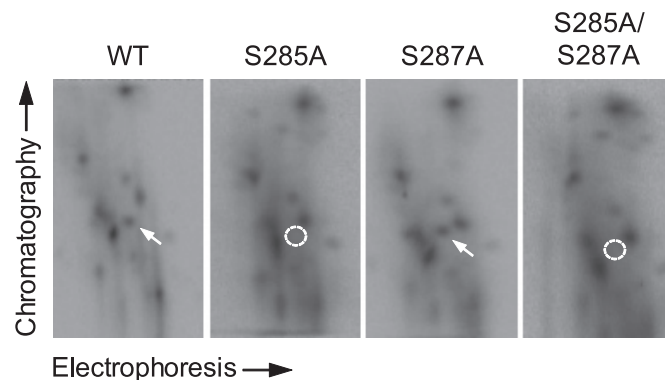


Figure 8. Phosphopeptide mapping analysis of WT, S285A, S287A, and S285A/S287A forms of lipin 1 β . The WT, S285A, or S285A/S287A form of lipin 1 β (0.1 μ g) was phosphorylated by 0.2 μ g of CKII with 50 μ M [γ - 32 P]ATP. The phosphorylated proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. The 32 P-labeled lipin 1 β proteins on the membrane were digested with TPCK-treated trypsin. The phosphopeptides produced by the proteolytic digestion were separated on cellulose TLC plates by electrophoresis (from left to right) in the first dimension and by chromatography (from bottom to top) in the second dimension. The white arrow indicates a phosphopeptide that is absent (dashed circle) in the maps of the S285A and S285A/S287A mutants. The data shown are representative of two independent experiments.

remaining putative phosphorylation sites (red color) (Fig. 7). No CKII activity was observed on the S285A peptide, whereas the activity on the S287A peptide was $82 \pm 4.8\%$ of that observed for the WT peptide. The phosphorylation of the S287A mutant peptide could be attributed to the phosphorylation of Ser-285, but it is unclear why no phosphorylation was observed for the S285A peptide because Ser-287 is available for phosphorylation.

The effects of the S285A, S287A, and S285A/S287A mutations on the phosphorylation of lipin 1 β by CKII

To confirm that Ser-285 and Ser-287 of lipin 1 β are the sites of phosphorylation by CKII, the serine residues, alone and in combination, were changed to alanine. The mutant lipin 1 β proteins purified after their expression in HeLa cells (Fig. 3) were treated with CKII and [γ - 32 P]ATP and then analyzed by SDS-PAGE and phosphorimaging. The S285A and S287A mutations did not show a major effect on the overall extent of lipin 1 β phosphorylation, suggesting that it is also phosphorylated on many other sites. To address this possibility, the CKII-phosphorylated WT lipin 1 β was digested with TPCK-treated trypsin, and the resulting peptides were separated by electrophoresis and TLC. This analysis showed multiple phosphopeptides from the CKII-phosphorylated lipin 1 β , indicating that the protein is phosphorylated on many sites (Fig. 8). The analysis of lipin 1 β with the PeptideCutter program (60) predicts that Ser-285 and Ser-287 should be contained in the same peptide. Consistent with this prediction, the phosphopeptide map of the S285A/S287A double mutant showed the loss (indicated by the dashed circle) of a single phosphopeptide (indicated by the white arrow in the phosphopeptide map of WT lipin 1 β) (Fig. 8). Moreover, this phosphopeptide was present (indicated by the white arrow) in the phosphopeptide map of the S287A mutant protein, which is consistent with the phosphorylation of Ser-285 (Fig. 8). However, the same phosphopeptide was missing (indicated by the dashed circle) in the phosphopeptide map of the S285A mutant (Fig. 8). This result suggested that the

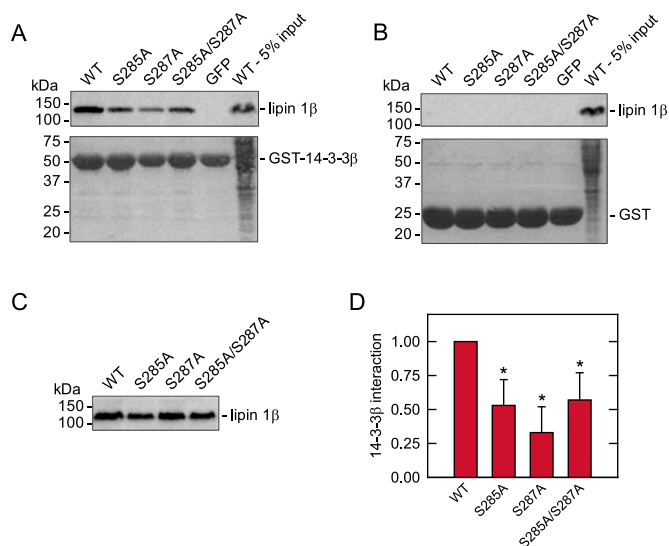


Figure 9. Effects of the phosphorylation-deficient mutations of lipin 1 β on its interaction with 14-3-3 β protein. GSH-Sepharose-bound GST-14-3-3 β (A) or GST (B) was incubated with the HEK-293T cell lysate (1 mg of protein) containing the WT or the S285A, S287A, or S285A/S287A mutant form of lipin 1 β at 4 °C for 2 h. Following the collection of the resin and extensive washing, the proteins were eluted and separated by SDS-PAGE. The resolved proteins were transferred to PVDF membrane and analyzed for lipin 1 β by immunoblotting with anti-FLAG antibodies. The lipin 1 β signals on the immunoblots were acquired with a Fuji LAS 4000 analyzer and quantified with Multi Gauge software. The GST-14-3-3 β and GST proteins on the blots were visualized by staining with Ponceau S. A blot showing the lipin 1 β input (5%) of the experiment is shown in C, and the quantification of the data is shown in D. The lipin 1 β signal that interacted with the GST-14-3-3 β protein was divided by the signal of the lipin 1 β input; the value obtained for each mutant protein was normalized to the WT control value that is arbitrarily set at 1.0. GFP was included as a negative control. The positions of lipin 1 β , GST-14-3-3, GST, and molecular mass standard are indicated. The data shown in A–C are representative of four independent experiments, whereas the data in D are the average of the four experiments \pm S.D. (error bars). *, $p < 0.05$ versus WT.

phosphorylation of Ser-287 was affected by the loss of phosphorylation on Ser-285.

The effects of the S285A, S287A, and S285A/S287A mutations on the interaction of lipin 1 β with 14-3-3 β protein

We examined the interaction of GST-tagged 14-3-3 β protein with the WT and S285A, S287A, and S285A/S287A forms of FLAG-tagged lipin 1 β . Consistent with the previous finding on the interaction of lipin 1 α with 14-3-3 β (46), lipin 1 β was shown to interact with GST-14-3-3 β protein (Fig. 9A). That the protein–protein interaction was specific is indicated by the inability of GST alone to interact with the lipin 1 β protein (Fig. 9B). The S285A, S287A, and S285A/S287A mutations caused reductions in the interaction of lipin 1 β with the 14-3-3 β protein by 47, 67, and 43%, respectively, when compared with the WT control (Fig. 9D). Whereas these reductions caused by the individual and combined mutations were significant, the mutational effects were not significantly different from each other (Fig. 9D).

Discussion

PA phosphatase has emerged as a key regulatory enzyme in eukaryotic lipid metabolism (8, 22, 61, 62). This is exemplified by the plethora of physiological defects and disease states imparted by the loss of its function to catalyze the conversion of

PA to DAG (8, 22, 61, 62). The posttranslational modification of phosphorylation largely governs whether the enzyme is associated with the cytoplasm apart from its substrate PA or with the membrane where the PA phosphatase reaction occurs (8, 22, 61, 62). Lipin 1 β PA phosphatase contains many serine and threonine residues that are phosphorylated (42, 47–51), but less than 10% of the sites can be ascribed to a specific protein kinase and signaling network (41, 42, 47, 52). In this work, a bioinformatics approach was taken to identify protein kinases responsible for the phosphorylation of lipin 1 β . Phosphoamino acid analysis showed that lipin 1 β is phosphorylated by CKII on the serine and threonine residues, and the enzymological analysis of CKII activity indicated that lipin 1 β is a *bona fide* substrate for the kinase.

The phosphopeptide mapping experiment of the CKII-phosphorylated lipin 1 β indicated that it is phosphorylated on multiple sites. To manageably identify the sites of phosphorylation, we focused on the serine-rich region of the protein. Péterfy *et al.* (46) have previously shown that the phosphorylation of the serine-rich region of lipin 1 α is required for its interaction with 14-3-3 proteins and retention in the cytoplasm. Because the phosphorylations of Ser-252, Ser-254, and Ser-260 within the serine-rich region are critical for the cytoplasmic localization of lipin 1 α (46) and the serine residues are putative CKII phosphorylation sites, we hypothesized that the corresponding residues, namely Ser-285, Ser-287, and Ser-293, in lipin 1 β are phosphorylated by CKII. The enzymological analysis of the WT and mutant versions of the lipin 1 β peptide corresponding to the serine-rich region led to the conclusion that Ser-285 and Ser-287 are CKII phosphorylation sites, with Ser-285 being the major site. Ser-293 was not identified as a CKII phosphorylation site by the assay employed in this study. On one hand, the phosphorylation assays with the lipin 1 β mutant peptide substrates (*e.g.* S285A and S287A) indicated that lack of phosphorylation on Ser-285 prevented the phosphorylation of Ser-287, whereas the lack of phosphorylation on Ser-287 did not affect the phosphorylation of Ser-285. On the other hand, CKII phosphorylated the lipin 1 β peptide S287, and in this peptide, Ser-285 is changed to an alanine. Yet the phosphopeptide-mapping experiments with the full-length lipin 1 β S285A and S287A mutants also indicated that the phosphorylation of Ser-285 affects the phosphorylation of Ser-287. The reason for this puzzlement is unclear.

Like lipin 1 α (46), the lipin 1 β associated with 14-3-3 β protein, and this association was attenuated by the CKII phosphorylation-deficient S285A, S287A, and S285A/S287A mutations. The phosphorylation of lipin 1 β by CKII did not affect its PA phosphatase activity *in vitro*. Thus, the effect of the CKII-mediated phosphorylation of lipin 1 β would be expected to inhibit PA phosphatase function by sequestration of the enzyme in the cytoplasm. The interaction of lipin 1 β with 14-3-3 β protein is expected to be more complex than just phosphorylation of a couple of sites (46), and thus, more work is needed to identify the protein kinase(s) that phosphorylate other residues within the serine-rich region of the protein. Additional work is also required to identify the CKII target sites outside the serine-rich region and clarify their role in regulating lipin 1 β function.

Phosphorylation of lipin 1 β PA phosphatase by CKII

To date, 44 serine/threonine residues have been identified as sites of phosphorylation in lipin 1 β (42, 47–51) (Fig. 2). Of the identified sites, mTORC1 phosphorylates Ser-106 and Ser-472 (42, 47), and CKI phosphorylates Ser-483 and Ser-487 (52). Insulin stimulation is followed by the phosphorylation of lipin 1 β by mTORC1 (41, 42, 47), whereas the phosphorylation by CKI causes lipin 1 β to interact with the SCF ^{β -TRCP} E3 ubiquitin ligase complex for its ubiquitination and degradation (52). These phosphorylations are hierarchical in nature; the phosphorylation by CKI requires prephosphorylation by mTORC1 (52).

The yeast Pah1 PA phosphatase also plays an important role in lipid metabolism and cell physiology (8, 63), and like lipin 1 β , the enzyme localization (*i.e.* cytoplasmic *versus* membrane) is governed by its phosphorylation (3, 64). Pah1 is subject to multiple (*i.e.* 34) phosphorylations (64–75), and some of the protein kinases involved have been identified and the sites mapped (76–80). These include protein kinases A (76) and C (77), the cyclin-dependent protein kinases Cdc28/CDK1 (78) and Pho85/CDK5 (79), and CKII (80). Phosphorylations by the cyclin-dependent protein kinases and protein kinase A sequester Pah1 in the cytoplasm (76, 78, 79, 81). The phosphorylations by Pho85/CDK5 and protein kinase A (76) also reduce the PA phosphatase activity of Pah1 (76, 79). The phosphorylation of Pah1 by CKII has little effect on PA phosphatase activity, but it inhibits the subsequent phosphorylation by protein kinase A (80). The phosphorylation by protein kinase C does not affect the location or catalytic activity of Pah1, but it facilitates the proteolytic degradation of the enzyme by the 20S proteasome (77, 82).

In this work, we focused on CKII because the kinase is essential to viability and cell cycle progression from yeast to humans (54–56, 83, 84), and it has been identified as one of the protein kinases that regulates yeast Pah1 PA phosphatase (80). In the context of lipin 1 β and signaling in mammalian cells, CKII is activated by insulin (85–87). One of the insulin-mediated targets of CKII is acetyl-CoA carboxylase (87), the enzyme that catalyzes the committed step in the synthesis of fatty acids (2). Fatty acids are the building blocks of triacylglycerol and membrane phospholipids, lipid molecules whose synthesis is also controlled by the activity of PA phosphatase (1–9). Like lipin 1 β , the phosphorylation of acetyl-CoA carboxylase by many protein kinases results in the attenuation of its function (88). Overall, the work reported here not only advances the understanding of lipin 1 β phosphorylation, it also sheds new light on the CKII-mediated regulation of lipid metabolism.

Experimental procedures

Materials

All chemicals were reagent grade or better. Dioleoyl PA was obtained from Avanti Polar Lipids. Protein assay reagents, electrophoresis reagents, protein molecular mass standards, and Coomassie Blue R-250 were from Bio-Rad. Leupeptin and pepstatin were purchased from Cayman Chemical. Cellulose TLC plates were from EMD Millipore. InstantBlue protein stain was purchased from Expedeon. Peptides were prepared by EZBiolab. GSH-Sepharose, pGEX4T3, pGEX4T3-14-3-3 β ,

PVDF membrane, and the enhanced chemifluorescence Western blotting reagent were purchased from GE Healthcare Life Sciences. CKII (α 2 β 2) was purchased from New England Biolabs (catalogue no. P6010S). PerkinElmer Life Sciences and National Diagnostics, respectively, were the sources of radiochemicals and scintillation counting supplies. Sigma-Aldrich was the source of BSA, nitrocellulose, Ponceau S, phosphoamino acid standards, PMSF, mouse anti-FLAG (DYKD-DDDK) M2 antibody (catalogue no. F3165), alkaline-conjugated goat anti-mouse antibody (catalogue no. A3562), and TPCK-treated trypsin. P81 phosphocellulose paper was from Whatman.

Preparation of lipin 1 β proteins

The S285A, S287A, and S285A/S287A mutations in mouse lipin 1 β were made using PCR site-directed mutagenesis in the pcDNA3 vector with FLAG-tagged lipin 1 β inserts. The mutagenesis was confirmed by DNA sequencing. The FLAG-tagged lipin 1 β cDNAs were placed into pAdTRACK-CMV; the shuttle vector was recombined with pAdEasy, and adenovirus was made by transformation of the linearized recombined plasmid in HEK-293 cells. The FLAG-tagged lipin 1 β proteins were expressed in HeLa cells by adenoviral infection and purified by affinity chromatography as described by Granade and Harris (58). Analysis of the proteins by SDS-PAGE indicated that they were purified to ~90% of homogeneity. The amounts of lipin 1 β proteins resolved in the SDS-polyacrylamide gel were quantified using BSA as a standard (58).

For the GST-14-3-3 β interaction studies, the FLAG-tagged WT or mutant forms of lipin 1 β were expressed in HEK-293T by transient transfection. HEK-293T cells in a 10-cm plate were transfected with 5 μ g of *pcDNA3-FLAG-Lpin1 β* (WT, S285A, S287A, or S285A/S287A). Three days post-transfection, the cells were scraped in 10 mM Na₂HPO₄ buffer (pH 7.4) containing 50 mM β -glycerophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, and 1 mM PMSF and centrifuged at 16,000 \times g for 10 min at 4 $^{\circ}$ C to obtain the clarified cell lysate.

Phosphorylation of lipin 1 β by CKII

CKII activity on lipin 1 β was measured by following the incorporation of the radioactive phosphate of [γ -³²P]ATP into the substrate. The assays were performed in triplicate for 15 min at 30 $^{\circ}$ C in a total volume of 20 μ l. The reaction mixture contained 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM β -mercaptoethanol, 0.2 μ g of CKII, 50 μ M [γ -³²P]ATP, and 0.1 μ g of lipin 1 β or 100 μ M lipin 1 β peptide unless otherwise indicated. The kinase reaction was terminated by the addition of 5 \times Laemmli sample buffer (89) and subjected to SDS-PAGE to separate ³²P-labeled lipin 1 β from [γ -³²P]ATP, and protein was transferred to a PVDF membrane unless otherwise indicated. Radioactively labeled lipin 1 β was visualized by phosphorimaging, and the extent of the phosphorylation was quantified by ImageQuant software. Alternatively, the reaction was terminated by spotting the mixture onto nitrocellulose paper, which was then washed three times with 75 mM phosphoric acid to remove unreacted radioactive ATP. The nitrocellulose paper was then subjected to scintillation counting. For the phosphor-

ylation of lipin 1 β peptides, the reaction was terminated by spotting the reaction mixture onto a P81 phosphocellulose paper, followed by phosphoric acid washing and scintillation counting. One unit of CKII activity was defined as 1 nmol/min, and specific activity was defined as the units per mg of CKII.

Analysis of phosphopeptides and phosphoamino acids

The ^{32}P -labeled lipin 1 β transferred to PVDF membrane was digested with TPCK-treated trypsin for phosphopeptide mapping and hydrolyzed with 6 N HCl at 110 °C for phosphoamino acid analysis (90–92). The tryptic digests were separated on the cellulose plates first by electrophoresis and then by TLC (90–92). The acid hydrolysates were mixed with standard phosphoamino acids and separated by two-dimensional electrophoresis on the cellulose plates. Radioactive phosphopeptides and phosphoamino acids were visualized by phosphorimaging analysis. Nonradioactive phosphoamino acid standards were visualized by ninhydrin staining.

Preparation of GST-14-3-3 β protein

Escherichia coli BL21 (DE3) pLysS cells were transformed with pGEX4T3 or pGEX4T3-14-3-3 β . The *E. coli* transformant was inoculated into 800 ml of lysogeny broth medium containing 100 $\mu\text{g}/\text{ml}$ ampicillin and grown to $A_{600\text{ nm}} = 0.5$. Transgene expression was induced with the addition of 0.5 mM isopropyl- β -D-1-thiogalactopyranoside, and cultures were incubated for an additional 2 h. The *E. coli* culture was harvested by centrifugation and lysed by sonication in PBS (pH 7.4) containing 0.5% Nonidet P-40, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, and 1 mM PMSF. The sonicate was centrifuged at $16,000 \times g$ for 10 min at 4 °C, and the supernatant was used as cell lysate. The lysate containing the overexpressed GST or GST-14-3-3 β (850 μg of protein) was incubated with GSH-Sepharose at 4 °C for 1 h, followed by washing of the resin three times with sonication buffer.

Analysis of lipin 1 β interaction with GST-14-3-3 β protein

GSH-Sepharose-bound GST-14-3-3 β or GST was incubated with the HEK-293T lysate (1 mg of protein) containing the WT or the S285A, S287A, and S285A/S287A mutant forms of lipin 1 β at 4 °C for 2 h with rotation and washed three times with 10 mM Na_2HPO_4 buffer (pH 7.4) containing 50 mM β -glycerophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, and 1 mM PMSF, followed by two additional washes with the same buffer containing 450 mM NaCl. Proteins were eluted from GSH-Sepharose by incubation in Laemmli's sample buffer (89) at 70 °C, followed by SDS-PAGE, transfer to PVDF membrane, and immunoblot analysis (93). Mouse anti-FLAG and alkaline-conjugated goat anti-mouse antibodies were used at dilutions of 1:1,000 and 1:30,000, respectively (94). GST-14-3-3 β or GST on the PVDF membrane was visualized by staining with Ponceau S. The images of immunoblot signals and Ponceau S staining were acquired with a Fuji LAS 4000 analyzer, and lipin 1 β proteins were quantified with Multi Gauge software.

PA phosphatase assay

PA phosphatase activity was measured by following the release of water-soluble P_i from chloroform-soluble PA using

the Triton X-100/PA-mixed micellar assay as described by Han and Carman (13). The reaction mixture contained 160 mM Tris-HCl (pH 7.5) buffer, 1 mM MgCl_2 , 10 mM 2-mercaptoethanol, 0.2 mM dioleoyl PA, 2 mM Triton X-100, and lipin 1 β protein in a total volume of 10 μl . Water-soluble P_i was measured with the malachite green–molybdate reagent at $A_{650\text{ nm}}$ (13, 95). Enzyme assays were conducted in triplicate, and the average S.D. value of the assays was $\pm 5\%$.

Analyses of data

SigmaPlot software was used to determine kinetic parameters according to the Michaelis–Menten equation. The statistical analyses were performed with SigmaPlot or GraphPad Prism software.

Author contributions—M. H., M. E. G., G.-S. H., T. E. H., and G. M. C. designed the study, analyzed the results, and prepared the manuscript. M. H. characterized the CKII phosphorylations of lipin 1 β and its peptides and identified the sites of phosphorylation. M. E. G. constructed the phosphorylation-deficient mutant forms of lipin 1 β for expression and purification and performed the 14-3-3 β interaction experiments with D. W. A. H. performed phosphorylation experiments on lipin 1 β that included the phosphoamino acid analysis and the phosphopeptide mapping experiments. J. M. K. performed the PA phosphatase assays on lipin 1 β phosphorylated by CKII.

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