Phosphorylation and dephosphorylation of Ca^{2+}/calmodulin-dependent protein kinase kinase β at Thr144 in HeLa cells

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Abbreviations

CaMKKβ, Ca^{2+}/CaM-dependent protein kinase kinase β; CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; PP1⁄2A, protein phosphatase 1⁄2A; OA, okadaic acid; AMPK, 5'AMP-activated protein kinase; PKB, protein kinase B; PKA, cAMP-dependent protein kinase; CDK5, cyclin-dependent kinase 5; GSK3, glycogen synthase kinase 3; DAPK, death-associated kinase; NRD, N-terminal regulatory domain.

Abstract

Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) acts as a regulatory kinase that phosphorylates and activates multiple downstream kinases including CaMKI, CaMKIV, 5'AMP-activated protein kinase (AMPK) and protein kinase B (PKB), resulting in regulation of wide variety of Ca^{2+}-dependent physiological responses under normal and pathological conditions. CaMKKβ is regulated by Ca^{2+}/calmodulin-binding, autophosphorylation, and transphosphorylation by multiple protein kinases including cAMP-dependent protein kinase (PKA). In this report, we found that phosphorylation of CaMKKβ is dynamically regulated by protein phosphatase/kinase system in HeLa cells. Global phosphoproteomic analysis revealed the constitutive phosphorylation at 8 Ser residues including Ser128, 132, and 136 in the N-terminal regulatory domain of rat CaMKKβ in unstimulated HeLa cells as well as inducible phosphorylation of Thr144 in the cells treated with a phosphatase inhibitor, okadaic acid (OA). Thr144 phosphorylation in CaMKKβ has shown to be rapidly induced by OA treatment in a time- and dose-dependent manner in transfected HeLa cells, indicating that Thr144 in CaMKKβ is maintained unphosphorylated state by protein phosphatase(s). We confirmed that *in vitro* dephosphorylation of pThr144 in CaMKKβ by protein phosphatase 2A and 1. We also found that the pharmacological inhibition of protein phosphatase(s) significantly induces CaMKKβ-phosphorylating activity (at Thr144) in HeLa cell lysates as well as in intact cells; however, it was unlikely that this activity was catalyzed by previously identified Thr144-kinases, such as AMPK and PKA. Taken together, these results suggest that the phosphorylation and dephosphorylation of Thr144 in CaMKKβ is dynamically regulated by multiple kinases/phosphatases signaling resulting in fine-tuning of the enzymatic property.

Key words; CaMKKβ, phosphorylation, dephosphorylation, PP1, PP2A, okadaic acid

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKKs) are known as activators for multiple protein kinases including CaMKI, CaMKIV, protein kinase B (PKB/Akt) and 5'AMP-activated protein kinase (AMPK) that phosphorylate the activation loop Thr residues to activate their catalytic efficiencies, resulting in the regulation of various physiological responses mediated by intracellular Ca^{2+} including neuronal, metabolic, and pathophysiological pathways [1-5]. CaMKK is composed of two isoforms (α and β) in mammals [6-8]. Previous studies demonstrated that CaMKKβ activates AMPK through phosphorylation of Thr172 in AMPKα, resulting in various metabolic and pathophysiological responses [9-11]. Especially, CaMKKβ-mediated signaling pathways are involved in cell growth of various cancers such as prostate [12], ovarian [4], and lung cancers [13] as well as in nonalcoholic fatty liver disease [14]. Similarly to other CaMKs, CaMKK is regulated by autoinhibitory mechanism and activated by Ca^{2+}/CaM-binding [15]. In addition to Ca^{2+}/CaM-binding, it has been demonstrated that CaMKKα is negatively regulated by phosphorylation with cAMP-dependent protein kinase (PKA), resulting in the recruitment of 14-3-3 proteins [16-18]. In contrast to CaMKKα, recombinant CaMKKβ has shown to be partially constitutively active because the N-terminal regulatory domain (NRD, residues 129-151) prevents the autoinhibitory mechanism of the enzyme [19]. It has been demonstrated that the phosphorylation of Ser129, Ser133, and Ser137 in the NRD of human CaMKKβ by cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 (GSK3) reduced autonomous activity [20]. Moreover, feedback phosphorylation of Thr144 in the same region by activated AMPK converts CaMKKβ into a Ca^{2+}/CaM-dependent enzyme [21], indicating that phosphorylation of the NRD suppresses the inhibitory effect of the region in the autoinhibitory mechanism. Recently, we have demonstrated that Thr144 in CaMKKβ is also rapidly phosphorylated by

β-adrenergic stimulation through cAMP/PKA signaling; this has been confirmed by *in vitro* phosphorylation of Thr144 by purified PKA [22].

In this study, we found that Thr144 in CaMKKβ is strictly maintained in the dephosphorylated state by protein phosphatases in resting HeLa cells and is phosphorylated by okadaic acid (OA)-activated protein kinase(s).

2. Materials and Methods

2.1. Materials

Recombinant rat CaMKKβ was expressed in *E. coli* BL21 Star (DE3) cells by using pET- rat CaMKKβ and purified by CaM-Sepharose and Q-Sepharose chromatography. pME-GST-rat CaMKKβ was constructed by PCR amplification of GST-rat CaMKKβ using pGEX-PreS-CaMKKβ [19] as a template, followed by subcloning into pME18s vector. Anti-phospho-CaMKKβ at Thr144 (clone A04) monoclonal antibody was generated as previously described [21]. Anti-CaMKKβ antibody was generated as previously described [23]. The catalytic subunit of cAMP-dependent protein kinase from bovine cardiac tissue was kindly provided by Dr. Yasuo Watanabe (Showa Pharmaceutical University). Okadaic acid was obtained from Wako Pure Chemical Industries (Osaka, Japan). Compound C and H-89 was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Cayman Chemical (Michigan, USA), respectively. Protein phosphatase 1 (#P7937) and protein phosphatase 2A (#14-111,) were purchased from Sigma-Aldrich (St. Louis, MO) and Upstate (Lake Placid, NY), respectively. All other chemicals were obtained from standard commercial sources.

2.2. Cell culture and transfection

HeLa cells were cultured in 6-well dishes or 10-cm dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 ^{o}C in 5% CO_2. HeLa cells in 6-well dishes were transfected with 2 μg (pME-CaMKKβ) expression plasmid using polyethylenimine "MAX" (Polysciences, Inc. Warrington, PA) according to the manufacturer's protocol. After 48-h culture, the cells were cultured with or without indicated concentrations of protein kinase inhibitors for 30 min, followed by treatment with okadaic acid for indicated time periods. Then, the cells were extracted with either 100 μL of lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 1% NP40, 10% Glycerol, 1:1000 protease inhibitor mixture (Nacalai Tesque, Kyoto Japan) or 1× SDS-PAGE sample buffer (100 μL), followed by immunoblot analyses (5–10 μL cell lysates).

2.3. Identification of phosphorylation sites in CaMKKβ by LC-MS/MS analysis.

HeLa cells in 10-cm dishes were transfected with 10 μg of GST-CaMKKβ (pME-GST-CaMKKβ) expression plasmid expressing and subsequently treated with 0.5 μM OA for 1 h, followed by washing with ice-cold PBS and, subsequently, extracted with 1 mL of ice-cold lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, and 0.05% Tween20). Cell lysates were subjected to purification with glutathione-sepharose resin (25 μL gel volume) and GST-CaMKKβ was eluted by addition of 1× SDS-PAGE sample buffer (50 μL) followed by subjecting to SDS-10% PAGE analysis. Lightly stained bands corresponding to GST-CaMKKβ (see Fig. 1A) on the gel were excised and subjected to protease digestion with a protease or protease mixture, including trypsin (Promega, Madison, USA), chymotrypsin (Roche Diagnostics GmbH, Mannheim, Germany), Glu-C (Roche Diagnostics GmbH), elastase (Promega), trypsin with Asp-N (Roche Diagnostics

GmbH), trypsin with chymotrypsin, trypsin with Glu-C or chymotrypsin with Asp-N. The following protease concentrations were used: 10 μg/mL trypsin, 17 μg/mL chymotrypsin, 10 μg/mL Glu-C, 4 μg/mL Asp-N, and 100 μg/mL elastase. Trypsin, Asp-N, and elastase were incubated at 37 °C, and chymotrypsin and Glu-C were incubated at 25 °C. The first and second digestions were incubated overnight and for 3 h, respectively. The digested peptides were analyzed by LC-MS/MS to identify the phosphorylation sites using an LCMS-IT-TOF instrument (Shimadzu, Kyoto, Japan) interfaced with a nano reverse-phase LC system (Shimadzu), as previously described [21]. The MS/MS data were acquired in the datum-dependent mode using the LC-MS solution software (Shimadzu) and converted into a single text file (containing the observed precursor peptide *m/z*, fragment ion *m/z*, and intensity values) using the Mascot Distiller (Matrix Science, London, UK). The MS/MS data were obtained independently and merged for the Mascot analysis (Supplemental figure S1). The following search parameters were used: database, rat CaMKKβ (587 amino acid residues); enzyme, all; variable modifications, carbamidomethyl (Cys), oxidation (Met), and phosphorylation (Ser/Thr).

2.4. Protein phosphatase assay

Recombinant CaMKKβ (293 μg) was phosphorylated with purified PKA (1μg) at 30 ^{o}C for 240 min as described previously [22], followed by purification with CaM-sepharose column chromatography, resulting in approximately 160 μg of phosphorylated CaMKKβ. Phosphorylated CaMKKβ (80 μg/mL) was incubated without or with protein phosphatase 2A (4.6 μg/mL) or protein phosphatase 1 (5–50 μg/mL) in a solution containing 40 mM Tris-HCl pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, and 0.1 mg/mL bovine serum albumin in the absence (for PP2A) or presence of 2 mM $MnCl_2$ (for PP1) at 30 ^{o}C for the

indicated time periods. Reactions were terminated by addition of an equal volume of 2× SDS-PAGE buffer, followed by immunoblot analysis.

2.5. In vitro Thr144-phosphorylation assay

HeLa cells in 6-well dishes was treated with 0.5 μM OA for indicated time periods, washed with ice-cold PBS and subsequently lysed with 50 μL of lysis buffer as described in 2.2. Recombinant CaMKKβ (1 μg) was incubated with 5 μL of HeLa cell extracts or purified catalytic subunit of PKA (10 ng) in a solution (20 μL) containing 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 100 μM ATP, and 100 nM OA in the absence or presence of protein kinase inhibitors at 30 °C for 30 min (10 min for PKA). The reaction was terminated by addition of an equal volume of 2× SDS-PAGE buffer, followed by immunoblot analysis using either anti-CaMKKβ or anti-pThr144 antibody.

2.6. Other methods

Immunoblot analysis was performed with the indicated primary antibodies and horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (GE Healthcare UK, Ltd.) as the secondary antibody. A chemiluminescent reagent (PerkinElmer Life Sciences, Waltham, MA, USA) was used for signal detection of immunoblots, followed by quantification of the immunoreactivity with ImageJ software [24]. Protein concentrations in samples were estimated by Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc.), using bovine serum albumin as a standard.

3. Results and Discussion

3.1. Global phosphorylation of CaMKKβ in HeLa cells

CaMKKβ has shown to be regulated by phosphorylation with multiple protein kinases in the NRD (residues 129–151, [19]) by GSK3 and CDK5 [20] and in the C-terminal region (at S511 in human CaMKKβ) by death-associated kinase (DAP kinase) [25] in transiently overexpressed cultured cells as well as *in vitro*. Recently, we identified Thr144 in the NRD of rat CaMKKβ by AMPK [21] and cAMP/PKA signaling in transiently expressed HeLa cells [22]. It has been shown that the Thr144 phosphorylation converts CaMKKβ into Ca^{2+}/CaM-dependent enzyme [21,22]. These studies also indicated that the Thr144 phosphorylation was very weak without stimulation, suggesting that the Thr144 is maintained unphosphorylated by protein phosphatase(s) in resting cells. Here, in order to examine the global Ser/Thr phosphorylation profile of CaMKKβ in intact cells, HeLa cells expressing GST-rat CaMKKβ were treated with 0.5 μM of a protein phosphatase inhibitor, okadaic acid (OA) for 1 hr (Fig. 1A) and then phosphorylation sites of purified GST-CaMKKβ were compared with those of untreated cells determined by LC-MS/MS analysis (Fig.1 C and Supplemental Figure S1). In the presence and absence of OA treatment, one phospho-Ser residue at N-terminal region (Ser22), three phosphorylation sites (Ser128, Ser132, Ser136) in the NRD, phospho-Ser494 in CaM-binding site and three phosphorylation sites (Ser510, Ser545, Ser571) in the C-terminal region were identified, indicating CaMKKβ is constitutively phosphorylated at multiple residues (Supplemental Figure S1) [20]. Furthermore, we found that OA treatment induces additional phosphorylation at Thr144 (Figure 1B, Supplemental Figure S1). These results are consistent with previous reports demonstrating that human CaMKKβ is phosphorylated at Ser129, Ser133, and Ser137 by CDK5/GSK3 in transiently overexpressed COS-7 cells [20] and Ser511 in

SH-SY5Y cells by DAPK [25] and Thr144 is maintained unphosphorylated state in unstimulated HeLa cells [21,22].

3.2. Phosphorylation of CaMKKβ at Thr144 in HeLa cells by okadaic acid treatment

To confirm the involvement of protein phosphatase(s) and kinase(s) in the regulation of CaMKKβ phosphorylation status, we analyzed the effect of OA on the Thr144 phosphorylation in transiently expressing rat CaMKKβ in HeLa cells by immunoblot analysis using an anti-pThr144 antibody. Thr144 phosphorylation of CaMKKβ was significantly induced by OA-treatment in a concentration-dependent (Fig. 2A) and a time-dependent (Fig. 2B) manner in transfected HeLa cells. This result is consistent with the fact that Thr144 phosphorylation is maintained at low level by dephosphorylation and rapidly induced by endogenous protein kinase(s) in HeLa cells. Previous studies demonstrated that the activated AMPK and PKA were capable of phosphorylating Thr144 in CaMKKβ *in vitro* as well as in cultured cells [21,22]. Therefore, we examined whether OA-induced Thr144 phosphorylation of CaMKKβ in the cells was catalyzed by AMPK or PKA by using pharmacological inhibitors (compound C, AMPK inhibitor and H-89, PKA inhibitor). As shown in Fig. 2C, treatment with 10 μM compound C did not significantly affect OA-induced Thr144 phosphorylation of CaMKKβ in HeLa cells (approximately 20% inhibition), indicating that AMPK is unlikely to be responsible for the Thr144 phosphorylation in the cells. In contrast, 10 μM H-89 treatment exhibited approximately 70% inhibition of the OA-induced CaMKKβ phosphorylation. Consistent with this result, dose-dependent effect of H-89 on the CaMKKβ phosphorylation in HeLa cells (Fig. 2D) indicated that 10 μM H-89 treatment exhibited approximately 60% inhibition of OA-induced Thr144 phosphorylation of CaMKKβ but 1 μM H-89 did not affect the phosphorylation (IC_{50} = 8 μM).

3.3. Induction of CaMKKβ phosphorylation activity in HeLa cells by okadaic acid treatment

To characterize the kinase activity in HeLa cells responsible for OA-induced Thr144 phosphorylation of CaMKKβ, we incubated the extracts of HeLa cells, which is treated or not treated with 0.5 μM OA for various time points, with recombinant CaMKKβ in the presence of Mg-ATP, followed by analysis of Thr144 phosphorylation activity by immunoblotting using an anti-pThr144 antibody. As shown in Fig. 3A, Thr144-phosphorylating activity in HeLa cell extracts was shown to be weak in untreated cells, which were significantly induced by OA-treatment. This indicates that the CaMKKβ-kinase is activated by OA-treatment suggesting that the kinase is downregulated by protein phosphatase(s). This result is in good agreement with the OA-induced phosphorylation at Thr144 in CaMKKβ in HeLa cells (Fig. 2A and B). Treatment of HeLa cells with compound C and H-89 as shown in Fig. 2C and D suggested that the OA-induced Thr144 phosphorylation in HeLa cells is catalyzed by H-89 sensitive kinases but it unlikely that AMPK catalyzes Thr144 phosphorylation. Therefore, we tested the effect of H-89 on the OA-induced Thr144-phosphorylation activity in HeLa cell extracts. As shown in Fig. 3B, H-89 up to 1 μM was incapable of inhibiting CaMKKβ-kinase activity in HeLa cell lysate and 10 μM of H-89 exhibited approximately 60% inhibition with an IC_{50} value of 9 μM, consistent with the effect of H-89 on OA-induced CaMKKβ Thr144 phosphorylation in HeLa cells as shown in Fig. 2C and D. To test whether the H-89 sensitive CaMKKβ-kinase in HeLa cell extracts is identical to PKA, we examined the effect of H-89 on the phosphorylation of CaMKKβ at Thr144 by purified PKA catalytic subunit (Fig. 3B *insert*). In contrast to the effect of H-89 on OA-induced CaMKKβ-kinase activity in HeLa cell extracts as shown in Fig. 3B, PKA activity was completely inhibited by 1 μM of H-89 with an IC_{50} value of 0.5 μM, which was

>10-fold more sensitive to H-89 than the OA-induced CaMKKβ-kinase activity in HeLa cells. It is noteworthy that compound C was incapable of inhibiting the CaMKKβ-kinase activity in HeLa cell extracts up to 10 μM (Supplemental Figure S2). These results indicate that the pharmacological inhibition of protein phosphatase(s) induces the CaMKKβ-phosphorylating activity (at Thr144) in HeLa cell lysates as well as in intact cells; however, it was unlikely that this activity was catalyzed by previously identified Thr144-kinases such as AMPK and PKA [21,22].

3.4. Dephosphorylation of CaMKKβ at Thr144 *in vitro*

Finally, to confirm the dephosphorylation of Thr144 in CaMKKβ by OA-sensitive protein phosphatases, *in vitro* dephosphorylation assay of CaMKKβ by protein phosphatase 2A (PP2A, Fig. 4A) and protein phosphatase 1 (PP1, Fig. 4B) was performed. By using recombinant CaMKKβ that had been phosphorylated at Thr144 by PKA [22] as a substrate, both protein phosphatases were capable of dephosphorylating Thr144 in CaMKKβ. It is noteworthy that the complete dephosphorylation of Thr144 within 60 min requires 10 times higher amount of PP1 (50 μg/mL, Fig. 4 B) than PP2A (4.6 μg/mL, Fig. 4A).

In this report, we found that Thr144 in CaMKKβ was strictly maintained in the dephosphorylated state in HeLa cells and phosphorylated by H-89 sensitive protein kinase(s) that were activated by pharmacological inhibition of the protein phosphatase activity in the cells whereas multiple Ser residues are constitutively phosphorylated in the cells. H-89 was reported not to be a specific inhibitor for PKA but capable of inhibiting multiple protein kinases including mitogen- and stress-activated protein kinase 1, p70 ribosomal protein S6 kinase 1 and Rho-dependent protein kinase II [26]. Therefore, our

pharmacological analyses suggest that it is unlikely that AMPK or PKA are responsible for OA-induced Thr144 phosphorylation in HeLa cells, even though these proteins were previously identified as CaMKKβ (Thr144) kinases [21,22]. Previous studies demonstrated that the Thr144 phosphorylation converted CaMKKβ into Ca^{2+}/CaM-dependent enzyme [21,22]. Taken together, these results may indicate that CaMKKβ is dynamically regulated by phosphorylation/dephosphorylation at Thr144 in the cells to fine-tune the enzymatic regulation.

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References

[1] G.A. Wayman, Y.S. Lee, H. Tokumitsu, A.J. Silva, T.R. Soderling

Calmodulin-kinases: modulators of neuronal development and plasticity

Neuron, 59 (2008), pp. 914-931, 10.1016/j.neuron.2008.08.021

[2] S. Takemoto-Kimura, K. Suzuki, S.I. Horigane, S. Kamijo, M. Inoue, M. Sakamoto, H. Fujii, H. Bito

Calmodulin kinases: essential regulators in health and disease

J Neurochem, 141 (2017), pp. 808-818, 10.1111/jnc.14020

[3] S. Yano, H. Tokumitsu, T.R. Soderling

Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway

Nature, 396 (1998), pp. 584-587, 10.1038/25147

[4] A.M. Gocher, G. Azabdaftari, L.M. Euscher, S. Dai, L.G. Karacosta, T.F. Franke, A.M. Edelman

Akt activation by Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2) in ovarian cancer cells

J Biol Chem, 292 (2017), pp. 14188-14204, 10.1074/jbc.M117.778464

[5] K.L. Marcelo, A.R. Means, B. York

The Ca^{2+}/Calmodulin/CaMKK2 Axis: Nature's Metabolic CaMshaft

Trends Endocrinol Metab, 27 (2016), pp. 706-718, 10.1016/j.tem.2016.06.001

[6] H. Tokumitsu, H. Enslen, T.R. Soderling

Characterization of a Ca^{2+}/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase

J Biol Chem, 270 (1995), pp. 19320-19324, 10.1074/jbc.270.33.19320

[7] T. Kitani, S. Okuno, H. Fujisawa

Molecular cloning of Ca^{2+}/calmodulin-dependent protein kinase kinase β

J Biochem, 122 (1997), pp. 243-250, 10.1093/oxfordjournals.jbchem.a021735

[8] K.A. Anderson, R.L. Means, Q.H. Huang, B.E. Kemp, E.G. Goldstein, M.A. Selbert, A.M. Edelman, R.T. Fremeau, A.R. Means

Components of a calmodulin-dependent protein kinase cascade. Molecular cloning, functional characterization and cellular localization of Ca^{2+}/calmodulin-dependent protein kinase kinase β

J Biol Chem, 273 (1998), pp. 31880-31889, 10.1074/jbc.273.48.31880

[9] A. Woods, K. Dickerson, R. Heath, S.P. Hong, M. Momcilovic, S.R. Johnstone, M. Carlson, D. Carling

Ca^{2+}/calmodulin-dependent protein kinase kinase-β acts upstream of AMP-activated protein kinase in mammalian cells

Cell Metab, 2 (2005), pp. 21-33, 10.1016/j.cmet.2005.06.005

[10] S.A. Hawley, D.A. Pan, K.J. Mustard, L. Ross, J. Bain, A.M. Edelman, B.G. Frenguelli, D.G. Hardie
Calmodulin-dependent protein kinase kinase-β is an alternative upstream kinase for AMP-activated protein kinase

Cell Metab, 2 (2005), pp. 9-19, 10.1016/j.cmet.2005.05.009

[11] R.L. Hurley, K.A. Anderson, J.M. Franzone, B.E. Kemp, A.R. Means, L.A. Witters
The Ca^{2+}/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases

J Biol Chem, 280 (2005), pp. 29060-29066, 10.1074/jbc.M503824200

[12] C.E. Massie, A. Lynch, A. Ramos-Montoya, J. Boren, R. Stark, L. Fazli, A. Warren, H. Scott, B. Madhu, N. Sharma, H. Bon, V. Zecchini, D.M. Smith, G.M. Denicola, N. Mathews, M. Osborne, J. Hadfield, S. Macarthur, B. Adryan, S.K. Lyons, K.M. Brindle, J. Griffiths, M.E. Gleave, P.S. Rennie, D.E. Neal, I.G. Mills
The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis

EMBO J, 30 (2011), pp. 2719-2733, 10.1038/emboj.2011.158

[13] L. Jin, J. Chun, C. Pan, A. Kumar, G. Zhang, Y. Ha, D. Li, G.N. Alesi, Y. Kang, L. Zhou, W.M. Yu, K.R. Magliocca, F.R. Khuri, C.K. Qu, C. Metallo, T.K. Owonikoko, S. Kang
The PLAG1-GDH1 Axis Promotes Anoikis Resistance and Tumor Metastasis through CamKK2-AMPK Signaling in LKB1-Deficient Lung Cancer

Mol Cell, 69 (2018), pp. 87-99 e87, 10.1016/j.molcel.2017.11.025

[14] B. York, F. Li, F. Lin, K.L. Marcelo, J. Mao, A. Dean, N. Gonzales, D. Gooden, S. Maity, C. Coarfa, N. Putluri, A.R. Means
Pharmacological inhibition of CaMKK2 with the selective antagonist STO-609 regresses NAFLD

Sci Rep, 7 (2017), pp. 11793, 10.1038/s41598-017-12139-3

[15] H. Tokumitsu, M. Muramatsu, M. Ikura, R. Kobayashi

Regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase kinase

J Biol Chem, 275 (2000), pp. 20090-20095, 10.1074/jbc.M002193200

[16] G.A. Wayman, H. Tokumitsu, T.R. Soderling

Inhibitory cross-talk by cAMP kinase on the calmodulin-dependent protein kinase cascade

J Biol Chem, 272 (1997), pp. 16073-16076, 10.1074/jbc.272.26.16073

[17] M. Matsushita, A.C. Nairn

Inhibition of the Ca^{2+}/calmodulin-dependent protein kinase I cascade by cAMP-dependent protein kinase

J Biol Chem, 274 (1999), pp. 10086-10093, 10.1074/jbc.274.15.10086

[18] M.A. Davare, T. Saneyoshi, E.S. Guire, S.C. Nygaard, T.R. Soderling

Inhibition of calcium/calmodulin-dependent protein kinase kinase by protein 14-3-3

J Biol Chem, 279 (2004), pp. 52191-52199, 10.1074/jbc.M409873200

[19] H. Tokumitsu, M. Iwabu, Y. Ishikawa, R. Kobayashi

Differential regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase kinase isoforms

Biochemistry, 40 (2001), pp. 13925-13932, 10.1021/bi010863k

[20] M.F. Green, J.W. Scott, R. Steel, J.S. Oakhill, B.E. Kemp, A.R. Means

Ca^{2+}/Calmodulin-dependent protein kinase kinase beta is regulated by multisite phosphorylation

J Biol Chem, 286 (2011), pp. 28066-28079, 10.1074/jbc.M111.251504

[21] A. Nakanishi, N. Hatano, Y. Fujiwara, A. Sha'ri, S. Takabatake, H. Akano, N. Kanayama, M. Magari, N. Nozaki, H. Tokumitsu

AMP-activated protein kinase-mediated feedback phosphorylation controls the Ca^{2+}/calmodulin (CaM) dependence of Ca^{2+}/CaM-dependent protein kinase kinase β

J Biol Chem, 292 (2017), pp. 19804-19813, 10.1074/jbc.M117.805085

[22] S. Takabatake, S. Ohtsuka, T. Sugawara, N. Hatano, N. Kanayama, M. Magari, H. Sakagami, H. Tokumitsu

Regulation of Ca^{2+}/calmodulin-dependent protein kinase kinase β by cAMP signaling

Biochim Biophys Acta Gen Subj, 1863 (2019), pp. 672-680, 10.1016/j.bbagen.2018.12.012

[23] H. Sakagami, M. Umemiya, S. Saito, H. Kondo

Distinct immunohistochemical localization of two isoforms of Ca^{2+}/calmodulin-dependent protein kinase kinases in the adult rat brain

Eur J Neurosci, 12 (2000), pp. 89-99, 10.1046/j.1460-9568.2000.00883.x

[24] C.A. Schneider, W.S. Rasband, K.W. Eliceiri

NIH Image to ImageJ: 25 years of image analysis Nat Methods, 9 (2012), pp. 671-675, 10.1038/nmeth.2089

[25] A.M. Schumacher, J.P. Schavocky, A.V. Velentza, S. Mirzoeva, D.M. Watterson

A calmodulin-regulated protein kinase linked to neuron survival is a substrate for the calmodulin-regulated death-associated protein kinase

Biochemistry, 43 (2004), pp. 8116-8124, 10.1021/bi049589v

[26] S.P. Davies, H. Reddy, M. Caivano, P. Cohen

Specificity and mechanism of action of some commonly used protein kinase inhibitors

Biochem J, 351 (2000), pp. 95-105, 10.1042/0264-6021:3510095

Figure Legends

Figure 1. Phosphorylation profile of rat CaMKKβ expressed in HeLa cells. **A**. GST-CaMKKβ (rat) expressed in HeLa cells that were treated (+) or not treated (−) with 0.5 μM okadaic acid (OA) for 1 h was partially purified by glutathione-sepharose resin as described in the "Materials and Methods", followed by SDS-PAGE analysis (25 μL each sample). Subsequently, protein bands corresponding to GST-CaMKKβ (*arrow*) were excised and subjected to LC-MS/MS analysis. The molecular mass in kDa is indicated on the *left*. **B**. The phosphorylation of Thr144 was analyzed through LC-MS/MS of the singly charged ion for peptides containing residues 142–148. The observed b- and y-ion fragment series generated by collision-induced dissociation are indicated above and below the peptide sequences (see Supplemental Figure S1). **C**. Schematic representation of the phosphorylation sites identified by LC-MS/MS analysis (see Supplemental Figure S1) in GST-CaMKKβ (rat) expressed in HeLa cells that were treated (+) or not treated (−) with 0.5 μM okadaic acid for 1 h as shown in *panel A*. NRD, N-terminal regulatory domain; AID/CaM, autoinhibitory and calmodulin-binding domain.

Figure 2. Induction of Thr144 phosphorylation in CaMKKβ expressed in HeLa cells by okadaic acid (OA) treatment. HeLa cells transfected with rat CaMKKβ expression vector were treated with various concentrations (0–0.5 μM) of OA for 1 h (**A**) or 0.1 μM of OA for the indicated time periods (**B**). HeLa cells transfected with rat CaMKKβ expression vector were incubated without (−) or with (+) 10 μM of compound C (CC) or 10 μM of H-89 (**C**) or with various concentrations (0–50 μM) of H-89 (**D**) for 30 min, and subsequently treated with 0.5 μM OA for 1h. After lysis of the cells with 1× SDS-PAGE sample buffer (100 μL), 10 μL of the cell lysates (duplicate) were subjected to immunoblot analysis using either

anti-phosphoThr144 antibody or anti-CaMKKβ antibody. The phosphorylation level of CaMKKβ at Thr144 was quantitated as duplicate experiments (**A**, **B**, **C**) and expressed as a percentage of the average value of the cells treated with 0.5 μM OA for 1 h without kinase inhibitors (**A**, **C**, **D**) or 0.1 μM OA for 5 h (**B**). **C** *insert*; (1) −OA, (2) + 0.5 μM OA, (3) + 0.5 μM OA, + 10 μM compound C (CC), (4) + 0.5 μM OA, + 10 μM H-89, (5) + 0.5 μM OA, + 10 μM CC/H-89. Arrows (pThr^{144}) in *insert panels* (**A**, **B**) and asterisks in *insert panels* (**C**, **D**) indicate phosphoCaMKKβ (at Thr144). Asterisks in *insert panels* (**A**, **B**) indicate CaMKKβ. The molecular mass in kDa is indicated on the *left* (*insert panels*). The data are representative of at least two independent experiments.

Figure 3. CaMKKβ phosphorylation (at Thr144) activity in HeLa cells. **A.** Lysates of HeLa cells that were not treated (0 min) or treated with 0.5 μM of okadaic acid (OA) for indicated time periods (10–90 min) were incubated with recombinant CaMKKβ (1 μg) in the presence of Mg-ATP for 30 min, followed by immunoblot analysis for detection of Thr144 phosphorylation (*upper panel*) and CaMKKβ (*lower panel*). The phosphorylation level of CaMKKβ at Thr144 was quantitated as duplicate experiments and expressed as a percentage of the average value of phosphorylated CaMKKβ with OA-treatment for 60 min. **B**. Lysates of HeLa cells treated with 0.5 μM of OA for 60 min or catalytic subunit of PKA (*insert panel*, Supplemental Figure S3) were assayed for CaMKKβ kinase activity as described in *panel A* in the presence of various concentrations (0–10 μM) of H-89. Thr144 phosphorylation activities were detected by immunoblot analysis using anti-pThr144 antibody (*upper panel*), quantitated as duplicate experiments and expressed as a percentage of the average value in the absence of H-89. Arrows (pThr^{144}) indicate

phosphoCaMKKβ (at Thr144). The molecular mass in kDa is indicated on the *left* (*upper panels*). The data are representative of at least two independent experiments.

Figure 4. Dephosphorylation of Thr144 in CaMKKβ by protein phosphatases. **A**. Recombinant CaMKKβ phosphorylated by PKA was incubated without (−PP2A, 0 min) or with protein phosphatase 2A (+PP2A) for indicated time periods (5–60 min) followed by terminating the reaction by addition of 2x SDS-PAGE sample buffer as described in the "Materials and Methods". **B**. Recombinant CaMKKβ phosphorylated by PKA was incubated without (0 μg) or with indicated amount of protein phosphatase 1 (5–50 μg) for 60 min followed by terminating the reaction as described in panel A. Reaction mixture (10 μl) were subjected to immunoblot analysis using either an anti-phosphoThr144 antibody (*upper panels*) or anti-CaMKKβ antibody (*lower panels*). The phosphorylation level of CaMKKβ at Thr144 was quantitated as duplicate experiments and expressed as a percentage of the average value of phosphorylated CaMKKβ without protein phosphatase treatment. Arrows ($pThr^{144}$) in *upper panels* (**A**, **B**) indicate phosphoCaMKKβ (at Thr144). The molecular mass in kDa is indicated on the *left* (*upper panels*). The data are representative of at least three independent experiments.

Figure 1

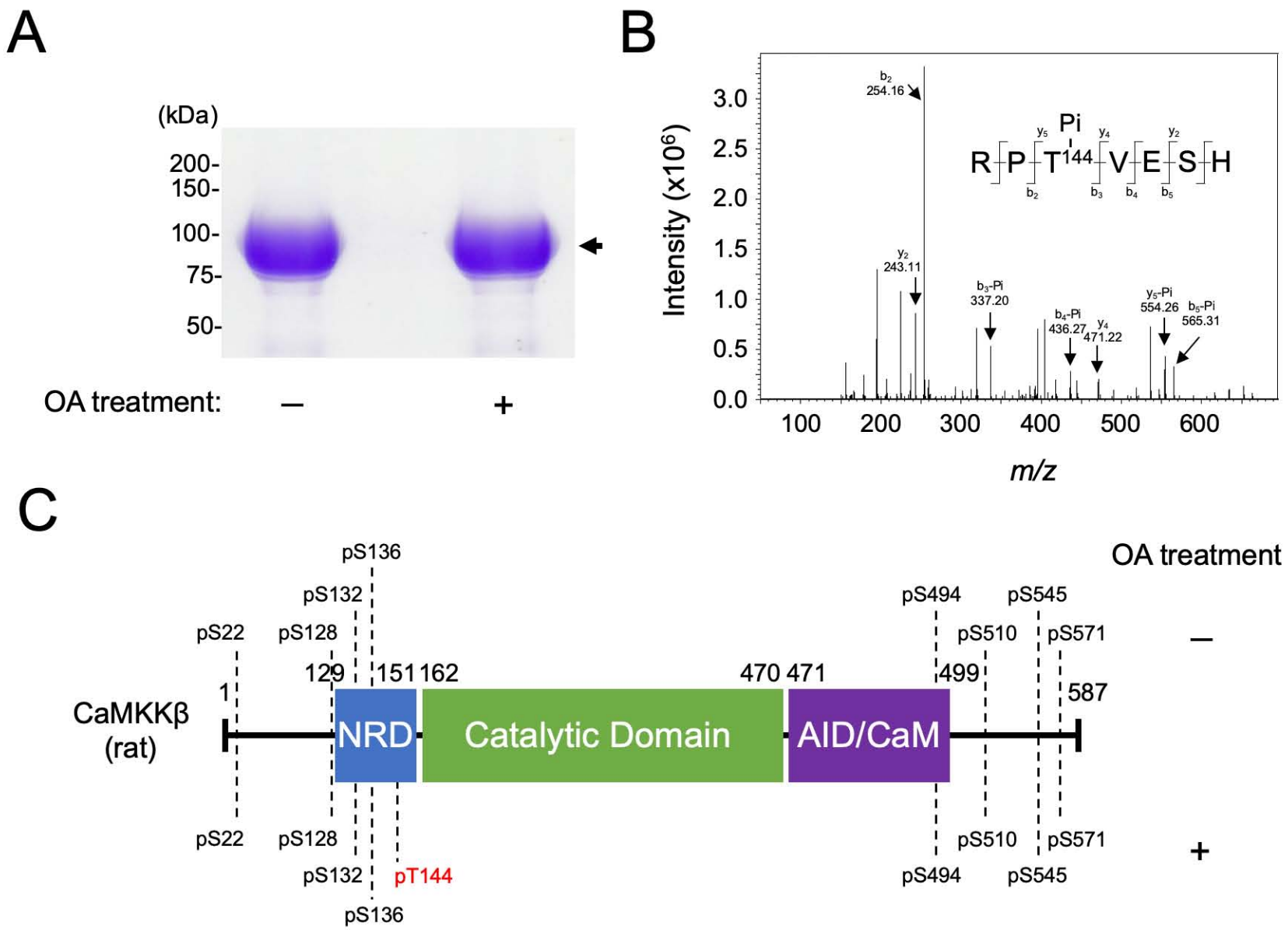
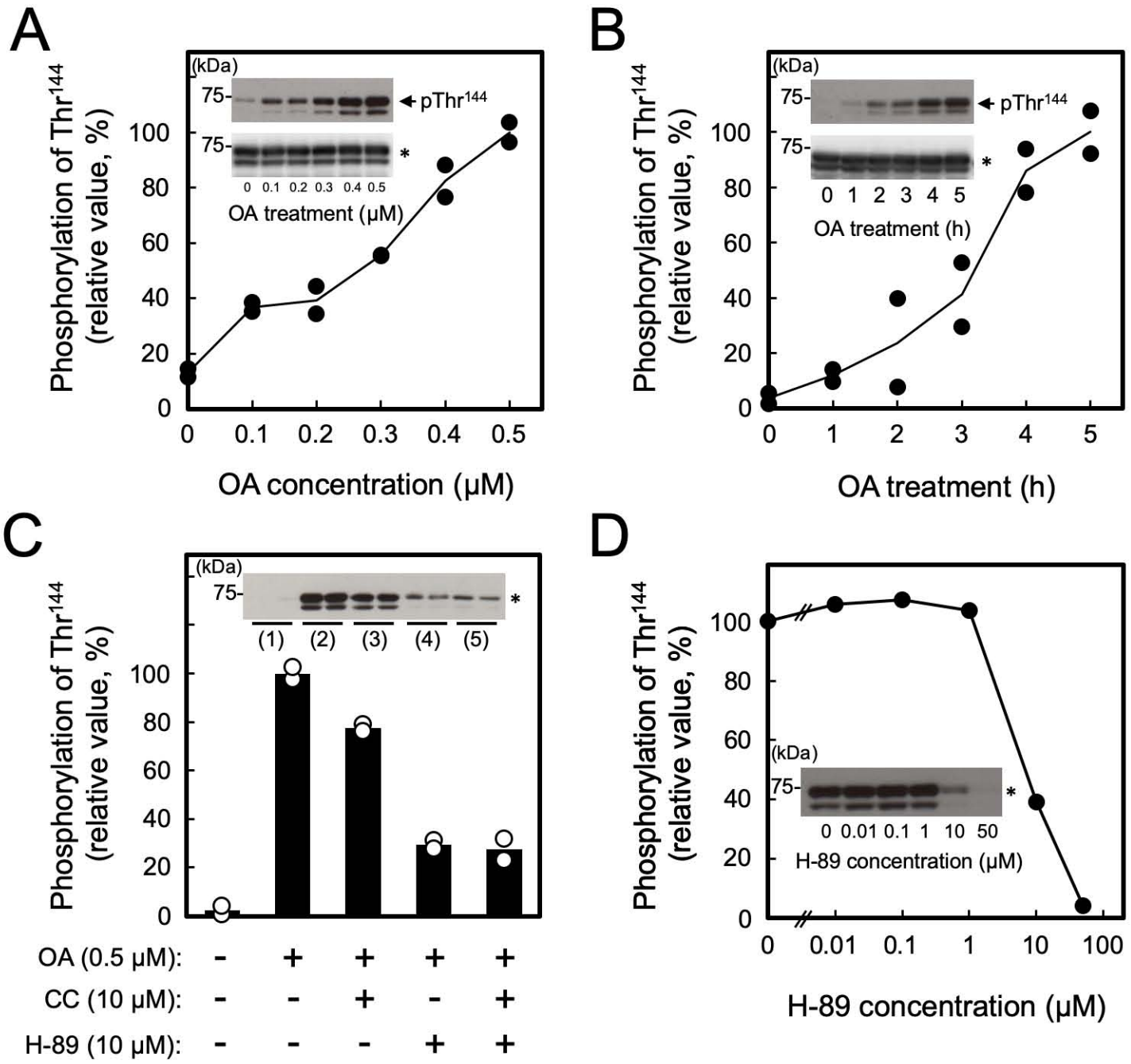
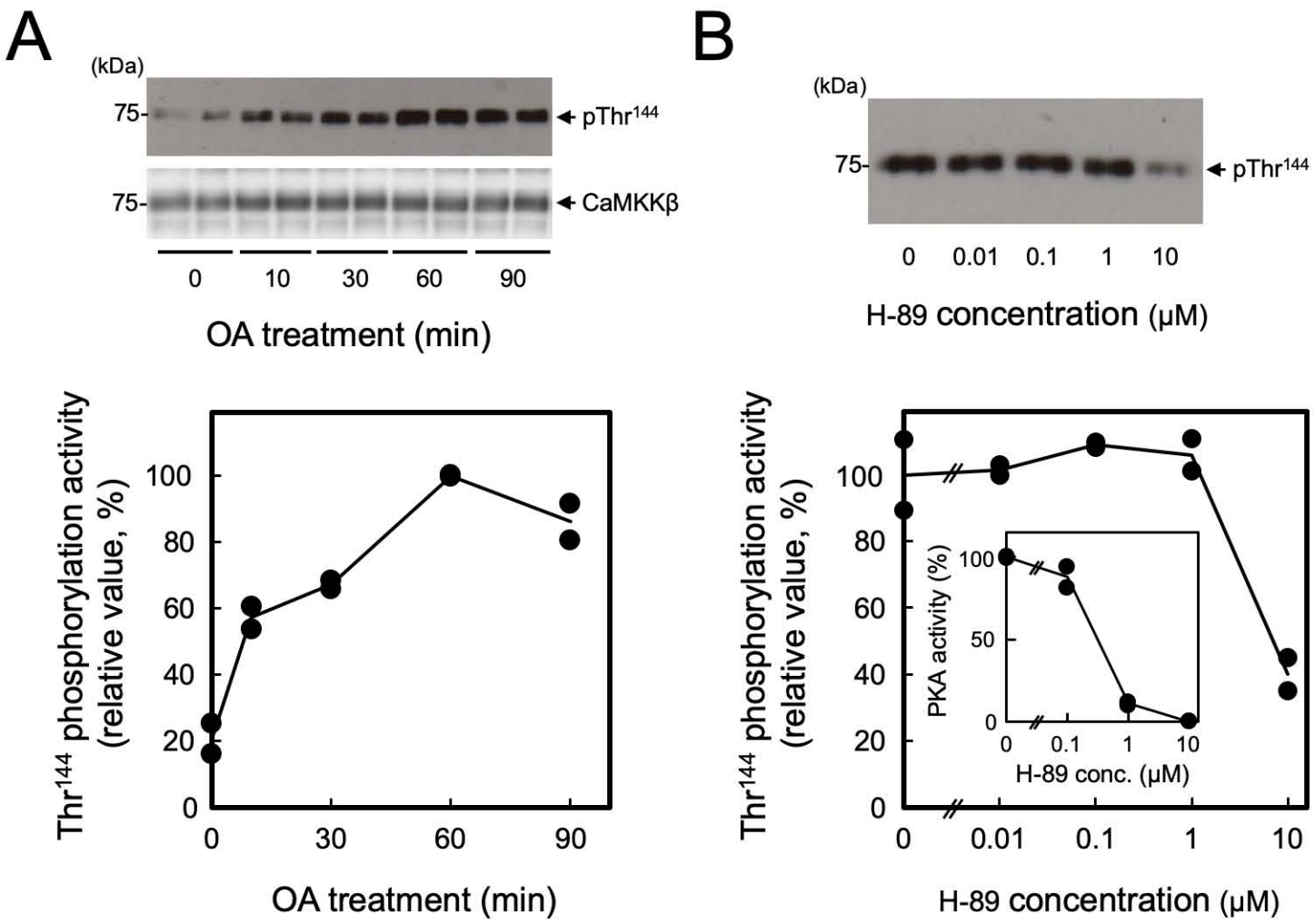
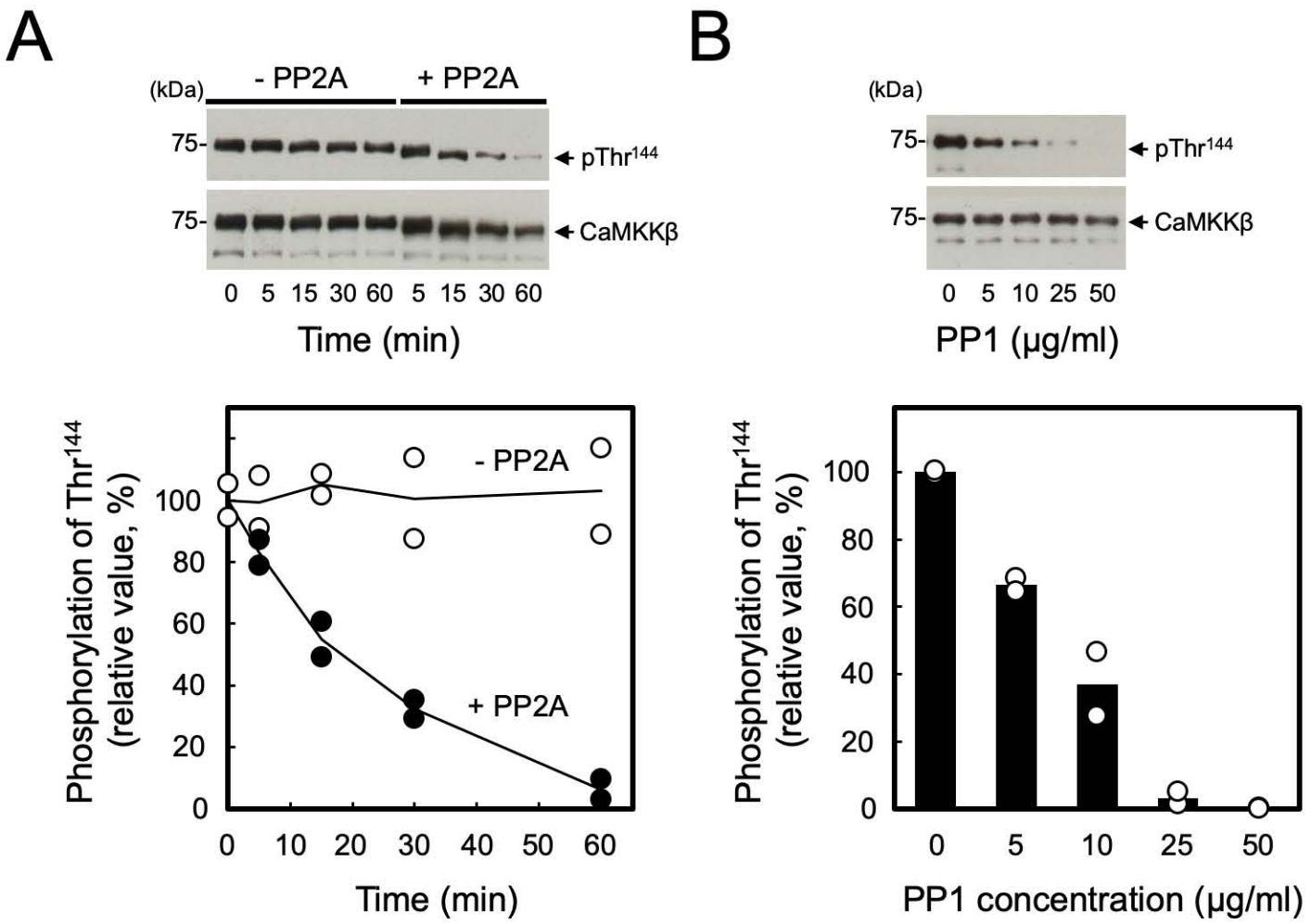

A
(kDa)
200-
150-
100-
75-
50-
OA treatment:
–
+
B
Intensity (x10^6)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100
200
300
400
500
600
m/z
b2 254.16
y2 243.11
b3-Pi 337.20
b4-Pi 436.27
y4 471.22
y5-Pi 554.26
b5-Pi 565.31
Pi
R P T144 V E S H
C
OA treatment
CaMKKβ (rat)
1
129
151
162
470
471
499
587
NRD
Catalytic Domain
AID/CaM
pS22
pS128
pS132
pS136
pT144
pS494
pS510
pS545
pS571
–
+

Figure 2


A
(kDa)
75-
pThr144
75-
*
0 0.1 0.2 0.3 0.4 0.5
OA treatment (μM)
Phosphorylation of Thr144 (relative value, %)
OA concentration (μM)
B
(kDa)
75-
pThr144
75-
*
0 1 2 3 4 5
OA treatment (h)
Phosphorylation of Thr144 (relative value, %)
OA treatment (h)
C
(kDa)
75-
*
(1) (2) (3) (4) (5)
Phosphorylation of Thr144 (relative value, %)
OA (0.5 μM): - + + + +
CC (10 μM): - - + - +
H-89 (10 μM): - - - + +
D
(kDa)
75-
*
0 0.01 0.1 1 10 50
H-89 concentration (μM)
Phosphorylation of Thr144 (relative value, %)
H-89 concentration (μM)


A
(kDa)
75-
pThr144
75-
CaMKKβ
0
10
30
60
90
OA treatment (min)
Thr144 phosphorylation activity (relative value, %)
0
20
40
60
80
100
0
30
60
90
OA treatment (min)
B
(kDa)
75-
pThr144
0
0.01
0.1
1
10
H-89 concentration (μM)
Thr144 phosphorylation activity (relative value, %)
0
20
40
60
80
100
0
0.01
0.1
1
10
H-89 concentration (μM)
PKA activity (%)
0
50
100
0
0.1
1
10
H-89 conc. (μM)


A
(kDa)
- PP2A
+ PP2A
75-
pThr144
75-
CaMKKβ
0 5 15 30 60 5 15 30 60
Time (min)
Phosphorylation of Thr144 (relative value, %)
- PP2A
+ PP2A
0 10 20 30 40 50 60
Time (min)
B
(kDa)
75-
pThr144
75-
CaMKKβ
0 5 10 25 50
PP1 (μg/ml)
Phosphorylation of Thr144 (relative value, %)
0 5 10 25 50
PP1 concentration (μg/ml)