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碩士論文

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Master Thesis

蛋白激酶 SIK2 和蛋白磷酸水解酶 PP2A 和 PP1 的交互調控

Functional interaction between SIK2 and PP2A or PP1 phosphatase

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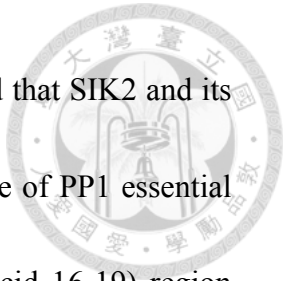
中文摘要

蛋白激酶 SIK2 屬於 AMPK 家族中的一員。目前已知 SIK2 在胰島素和葡萄醣代謝扮演重要角色，但其餘相關作用都還未知。實驗室目前已知 SIK2 的激酶活性會受到 p300/CBP 的乙醯化和 HDAC 的去乙醯化所調控。為研究 SIK2 之功能，從其交互作用的蛋白質著手是一常用的研究法；SIK2 是 AMPK 家族中唯一會和 p97/VCP 和蛋白磷酸水解酶 PP2A 有交互作用的成員。另外，SIK2 和 p97/VCP 的交互作用會促進內質網蛋白質降解。本研究主要針對 SIK2 和 PP2A 的功能及交互作用探討，同時也會討論 SIK2 和 PP1 的交互作用。我的主要目標是研究調控 SIK2-PP2A 複合體的形成的相關機制。SIK2 只會和完整三個次單元組成之具活性之 PP2A 結合且 PP2A 依然保有磷酸水解酶的活性。由於 SIK2 上的羧丁胺酸 Thr175 受到磷酸化也顯示在 SIK2-PP2A 複合體中的 SIK2 可能保有活性。此外，我觀察到當利用高濃度的 okadaic acid (OA)處理的 HEK293T 細胞，在蛋白質電泳當中，SIK2 會出現泳動遲至的現象。而磷酸化的 SIK2/T175 在高濃度 OA (大於 0.3 μM)處理下可以被偵測到。這個結果說明 PP1 可能會影響 SIK2 相關的磷酸化機制。實驗結果發現 SIK2 和其結合的 p97/VCP 都會與 PP1 有交互作用。PP1 與 SIK2 蛋白質的結合點位在 RVGF (胺基酸位置 16-19), 但與 p97/VCP 產生交互作用結合點尚待鑑定。最後，我也證明 SIK2 可能參與在 MEF2C 所調控的基因表現。

關鍵字： SIK2, PP2A, PP1, p97/VCP, RVxF motif, MEF2C 調控轉錄機制

Abstract

Salt-inducible kinase 2 (SIK2) is a member of AMPK family. Except for its roles in insulin signaling and glucose metabolism, the functions of SIK2 remain largely unknown. Our laboratory has demonstrated that the SIK2 kinase activity may be regulated by p300/CBP-mediated acetylation and HDAC6-induced deacetylation. SIK2 is the only member of the AMPK family capable of interacting with p97/VCP and protein phosphatase 2A (PP2A). Furthermore, interaction between SIK2 and p97/VCP was shown to facilitate ER-associated protein degradation (ERAD). In this thesis, I present results of physical and functional interactions between SIK2 and PP2A as well as protein phosphatase 1 (PP1). My major aim is to study the physiological cues responsible for regulation of SIK2 and PP2A complex formation. One of the major findings was that SIK2 interacts with PP2A holoenzyme. The elevated phosphorylation of SIK2 at Thr175 in SIK2-PP2A complex suggested that SIK2 activity was preserved in the complex. The kinase activity of SIK2-PP2A complex was further demonstrated by phosphorylation of a GST-syntide-2 substrate. In addition to PP2A, I have observed that when HEK293T cells were treated with high, but not low, concentration of okadaic acid (OA, ~0.3 μM vs. 0.1 μM), the mobility of SIK2 in SDS gel was retarded. The phosphorylated SIK2/T175 level of OA-treated sample is higher than that of control. These results suggest that PP1 inactivation may contribute to the mobility shift and



hyper-phosphorylation of SIK2. Further experiments have uncovered that SIK2 and its associated p97/VCP protein both interact with PP1. The docking site of PP1 essential for its binding to SIK2 has been identified in the RVGF (amino acid 16-19) region while the docking site(s) in p97/VCP remains to be determined. Finally, I have demonstrated that SIK2 plays regulatory functions in MEF2C-mediated gene expression.

Key Words: SIK2, PP2A, PP1, p97/VCP, RVxF motif, MEF2C-mediated transcription

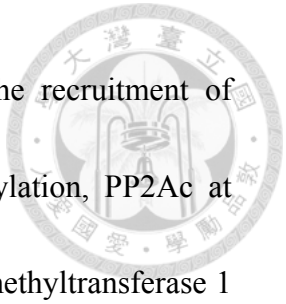
Contents

Introduction

Salt-inducible kinase 2 (SIK2) is a serine/threonine protein kinase which was first identified as a closely related member of the adipose-specific isoform of SIK1 [1,2]. Human SIK2 is a 926-amino acid protein consists of a protein kinase domain, an ubiqutin-binding like motif and a PKA phosphorylation site [3]. SIK2 belongs to member of the AMP-activated protein kinase (AMPK) family, SIK subfamily consisting SIK1, SIK2 and SIK3. AMPK family proteins are generally believed to have roles in metabolic regulation. Previous study showed that SIK2 mRNA is abundantly expressed in adipocytes. It regulates the insulin signal transduction by phosphorylating human IRS-1 at Ser794 [4]. SIK2 phosphorylates TORC2 at Ser171 resulting its being sequestered in the cytoplasm by 14-3-3 during insulin-modulated gluconeogenesis [5]. The phosphorylation of TORC2 also showed that SIK2 regulate insulin-induced PGC-1α and UCP-1 mRNA level and might involve in metabolism in brown adipocyte [6]. In addition, SIK2 phophorylated HAT coactivator p300 resulted in decreased ChREBP-mediated hepatic lipogenesis and steastosis [7]. The phosphorylation of TORC1 via SIK2 regulated the melanoenesis [8] and neuronal survival in mice [9]. SIK2 is also known to regulate mitosis and corticotrophin-releasing hormone transcription through phosphorylating C-Nap1 and TORC2, respectively [10,11]. The Lys53 acetylation inactivates SIK2 kinase activity and promotes autophagosome

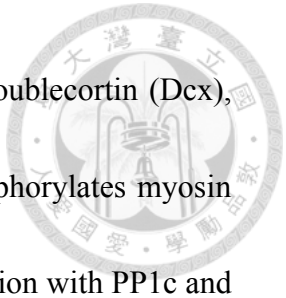
formation [12]. However, the mechanism of SIK2 in autophagy regulation remains to be studied.

Protein phosphatase 2 A (PP2A) is an evolutionarily conserved serine/threonine phosphatase. PP2A is a holoenzyme consists of three subunits, a scaffold A (PP2Aa), a catalytic C (PP2Ac), and a variable regulatory B (PP2Ab) subunit. Scaffolding subunit and catalytic subunit form a heterodimer core enzyme. Holoenzyme is formed upon recruitment of regulatory subunit the core enzyme. There are four distinct families of regulatory B subunits: B (B55/PR55), B' (B56/PR61), B'' (PR72/PR130), and B''' (PR93/PR110), and share a conserved binding domain interacting with PP2A A subunit [13-16]. PP2A have been found to be important in several cellular signal transduction pathways, such as cellular metabolism, cell differentiation, proliferation, meiosis, apoptosis, DNA transcription and translation [13-15,17]. In addition, PP2A regulates the functions of target proteins through reversible phosphorylation [18-20]. PP2A is proposed to be a tumor suppressor as evidenced by promotion of tumor growth when its phosphatase activity is inhibited and tumor regression when PP2A is overexpressed [21]. In cortex and hippocampus, the treatment of Ginsenoside Rb1 improved the learning and reversed the tau hyperphosphorylation level by regulating GSK3 and PP2A level [22]. Emi2 has been proved that involve in CSF-mediated metaphase arrest of eggs through the dephosphorylation by PP2A in vertebrate [23]. The tyrosine



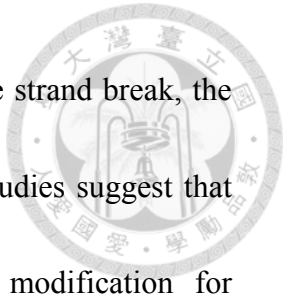
phosphorylation of PP2Ac at Tyr307 was reported to regulate the recruitment of regulatory B subunit [18]. In addition to regulation by phosphorylation, PP2Ac at Leu309 was performed reversible methylation by leucine carboxyl methyltransferase 1 (LCMT1) and demethylation by phosphatase methylesterase-1 (PME-1) [24-26]. Previous studies [27,28] and data [29] showed that PP2A interacted with SIK2 and CaMKI were negatively regulated by SIK2 and PP2A, suggested potential regulation of SIK2-PP2A complex in CaMKI pathway. My thesis focused on the interaction and function of SIK2 and PP2A complex.

During studying the phosphatase activity in SIK2-PP2A by pharmacological inhibition with okadaic acid, I serendipitously discovered that PP1 may participate in the regulation of kinase activity in SIK2-PP2A complex. PP1 is a serine/threonine phosphatase belongs to the phosphoprotein phosphatas (PPP) superfamily. PP1 contains two subunits, a catalytic subunit (PP1c) and a regulatory subunit. Most of regulatory proteins also known as PP1-interacting proteins (PIPs) interact with PP1 through a short conserved docking site, RVxF motif. Many PIPs function in protein synthesis and stress response, among others [30,31]. More than 70% of regulatory proteins are characterized as intrinsically disordered proteins (IDPs), such as spinophilin, MYPT1 and I-2, and the interaction of PP1 and PIPs may be involved in various functions [32]. PP1 is involved in the microtubule bundling during axonal



growth by spinophilin activation-dependent dephosphorylation of doublecortin (Dcx), microtubule-associated proteins (MAP) at Ser297 [33]. PKC phosphorylates myosin phosphatase target subunit 1 (MYPT1) and then promotes its interaction with PP1c and phosphorylated 20kDa light chain of myosin (LC_{20}). Up-regulation of phosphorylated LC_{20} regulates the smooth muscle contraction by inhibiting PP1 [34,35]. Furthermore, PP1 interacts with p54nrb and PSF via RVxF motif and regulate their transcriptional coexpression and alternative RNA splicing [36]. PP1 is also required for microtubule dynamics, glycogen synthesis, muscle contraction, cell cycle progression, mitosis, apoptosis and the regulation of membrane receptor [37-42]. In addition, PP1 regulates the spacing effect for memory in CREB pathway [43].

Previous results from our laboratory showed that SIK2 interacts with p97/VCP, a member of the type II AAA (ATPases associated with a variety of cellular activities) protein. The ATPase activity of p97/VCP is stimulated by SIK2-mediated phosphorylation at S770 [44]. p97/VCP plays an important role in control of cell cycle, DNA damage response, transcription, metabolism, autophagy and ERAD [45-50]. When mutants of the p97/VCP phosohorylation sites were overexpressed, cell proliferation was inhibited and apoptosis was induced. Furthermore, tyrosine phosphorylation of p97 is required for transitional ER assembly, T-cell activation and cell cycle-dependent nuclear localization [51-53]. Serine phosphorylation of p97/VCP



was demonstrated to regulate its recruitment to sites to DNA double strand break, the release of ubiquitinated proteins and cell survival [54-57]. These studies suggest that the phosphorylation of p97/VCP is important posttranslational modification for regulating its function. p97/VCP has two RVxF binding motif, and one of them, R159G mutant was found frequently in Amyotrophic Lateral Sclerosis (ALS) patients [58]. SIK2 and p97/VCP sequence contains RVxF motif, suggesting PP1 might associate and regulate their phosphorylation level and function.

In this study, I examined the regulation and physiological function of SIK2-PP2A complex. The complex formation of SIK2 and PP2A were affected by okadaic acid treatment. I demonstrated that PP2A holoenzyme is required for the formation of SIK2-PP2A complex. The phosphorylation of SIK2 at Thr175 and SIK2 kinase activity were preserved in SIK-PP2A complex. I also discovered that SIK2 and p97/VCP interact with PP1α through PP1α binding pocket. SIK2 may be involved in MEF2C-mediated gene expression.

Materials and methods

Antibodies, drugs and plasmids

Mouse monoclonal antibody against HA-tag was purchased from Sigma. Mouse monoclonal antibody against PP2Ac and PP2Aa were purchased from Cell Signaling. Mouse monocolonal anti-SIK2, anti-p97 and anti-α-tubulin, and rabbit polycolonal anti-actin, anti-SIK2/T175p, anti-p97/S770p and anti-Flag-tag antibodies were generated in our laboratory according to standard protocol. Okadaic acid and Nocodazole were purchased from Sigma. The DNA fragment of PP1 catalytic subunit was encoded from 293T cDNA. To construct Flag- and HA-tagged PP1α, PP1α coding region was excised using BamHI/EcoRV sites in the pCMV-Taq2B and BamHI/EcoRI sites in pcDNA 3/HA, respectively. The following primers were used in PCR:

PP1α -BamHI-F': 5'-AAA GGA TCC GCC ATG TCC GAC AGC GAG AA-3'

PP1α -EcoRV-R': 5'-GGG GAT ATC GGG CTA TTT CTT GGC TTT GGC-3'

PP1α -EcoRI-R': 5'-GGG GAA TTC GGG CTA TTT CTT GGC TTT GGC-3'

Site-directed mutation in plasmid

SIK2, p97 and PP1α mutation were generated by using pCMV-Flag-SIK2, pCMV-Flag-His-P97 and pcDNA-HA-PP1α as templates and using the QuikChange

site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions. The primers as follow:

SIK2-F19Y: 5'-GGTCCGGGTGGGGTACTACGACATCGAGG-3';

SIK2-V17A: 5'-CCGGTCCGGGCGGGGTTCTACGACATC-3';

SIK2-V17A/F19Y: 5'-CCGGTCCGGGCGGGGTACTACGACATCGAG-3';

p97-R159G: 5'-GGGTGGGATGGGTGCTGTGGAGTTCAAAG-3';

PP1α-C291R: 5'-GTGGACGAGACCCTCATGAGATCTTTCCAGATCC3'

Cell culture and transfection

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum and 100 unit/ml penicillin and streptomycin. Cultures were maintained at 37 °C with 5% CO_2. TurboFect (Fermentas) transfection of HEK293T cells was used according to manufacturer's instructions.

Lentivirus-shSIK2-mediated knockdown of SIK2

16 hours before lentivirus-shSIK2 infection, plate 0.5×10^5 of HEK293T cells in a well of 6-well plate in 2 ml medium. Lentivirus-shSIK2 in DMEM was added to the culture cells at MOI of 20 and incubated for forty-eight hours.

Protein extraction

HEK293T cell were rinsed with PBS and lysed with whole cell extraction (WCE) buffer (0.2 M NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 10 mM PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin A, 5 mM NaF, 1mM sodium vanadate and 1mM sodium butyrate). The lysate was kept on ice for 20 minutes, and then centrifuged at 15,000×g for 25 minutes at 4 °C.

Immunoblot analysis

Proteins in the lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to Hybond-C membrane (Amersham Bioscience). The membrane was blocked with 5% non-fat milk in PBST at room temperature for 30 minutes followed by probing with primary antibody at room temperature for 1 hour. After washed with PBST three times, the membrane was probed with HRP-conjugated secondary antibody at room temperature for 1 hour and washed with PBST three times. The membrane was incubated with HRP-ECL (PerkinElmer) and exposed to X-ray film (Kodak).

Immunoprecipitation

Flag-tagged, HA-tagged and His-tagged fusion protein were purified from WCE

buffer. The cell lysate was incubated with M2 beads, anti-HA beads and Ni-NTA beads (Sigma) for 90 minutes at 4 °C, respectively. After incubation, the beads were washed three times with WCE buffer. Boiled samples in SDS-PAGE sample buffer for 5 minutes, separated by SDS-PAGE and performed immunoblot analysis.

In vitro kinase assay

SIK2-PP2A complex was immunoprecipitated with HA beads. The precipitated protein complex was washed twice with kinase assay buffer (20 mM Tris-HCl, pH7.5, 10 mM $MgCl_2$, 1 mM DTT, 10 mM PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin A, 5 mM NaF, 1mM sodium vanadate and 1mM sodium butyrate). Kinase reaction was incubated with 1 mM ATP and 1 μl purified GST-syntide-2 at 30 °C for 30 minutes.

Reporter assay

The analysis was performed by using the Dual-Luciferase Reporter Assay (Promega) according to manufacturer's instructions. And the luciferase signal was detected by VICTOR X4 multilabel plate reader (PerkinElmer).

Statistical analysis

Data were analyzed by one-tailed Student's t-test. The mean and standard deviation (S.D.) were estimated from data and p-value <0.05 was considered significant.

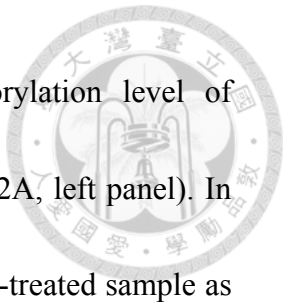
Results

SIK2 interacts with PP2A holoenzyme

To investigate the subunit involved in the interaction between PP2A and SIK2, I performed cotransfection of Flag-SIK2 and HA-PP2Ac into HEK293T cells. Cell-free extracts were prepared in the absence or presence of OA and used for immunoprecipitation experiments with M2 beads. OA treatment resulted in decreased levels of associated PP2Aa and PP2Ac subunits (Fig. 1A). These results suggest that the interaction between SIK2 and PP2A is impaired by OA. To find out whether PP2A holoenzyme is involved in its interaction with SIK2, I co-transfected Flag-PR55 (a PP2A B subunit) and His-SIK2 into HEK293T cells followed by immunoprecipitation with Ni-NTA or M2 beads. The results showed that SIK2 interacts with PR55-containing holoenzyme, The interaction was impaired when the transfected cells were treated with OA (Fig. 1B). These results suggest that PP2A holoenzyme is required for the formation of SIK2-PP2A complex and PP2A core enzyme.

SIK2 activity is preserved in SIK2-PP2A complex

To examine whether the formation of SIK2-PP2A complex affect SIK2 activity, I performed transfection of Flag-SIK2 into HEK293T cells followed by OA treatment



and immunoprecipitation. The results showed that the phosphorylation level of SIK2/T175 was higher in OA-treated than the control sample (Fig. 2A, left panel). In addition, the phosphorylation level of SIK2 was also elevated in OA-treated sample as shown by retardation of mobility in SDS-gel (Fig. 2A, right panel). When M2 beads bound SIK2-PP2A complex was incubated with OA, the phosphorylation level of SIK2/T175 was enhanced and the PP2Ac was slightly decreased (Fig. 2B). These data suggested that PP2A or associated phosphatase (e.g., PP1) activity in SIK2-PP2A complex might regulate the phosphorylation level of SIK2 at T175. To further confirm that SIK2 in SIK2-PP2A complex is active, I conducted *in vitro* kinase assay using purified GST-syntide-2 substrate and anti-HA immunoprecipitate prepared from HEK293T cells transfected with HA-PP2Ac and Flag-SIK2. The results showed that GST-syntide-2 is indeed phosphorylated by the kinase activity in SIK2-PP2A complex (Fig. 2C).

Interaction between SIK2 and PP1

An intriguing mobility shift of SIK2 was observed when HEK293T cells were treated with high (0.3 μM) but not low (0.1 μM) concentration of OA (Fig.3). The supershifted signal was detected by phosphospecific anti-SIK2-T175 antibody. These

results suggest that SIK2 was hyper-phosphorylated when treated with 0.3 μM OA. The results also showed that level of SIK2/T175p is elevated in 0.3 μM OA-treated sample (Fig. 3). PP1 is inhibited by OA if the concentration is higher than 0.3 μM. To determine the possible involvement of PP1 in regulating the phosphorylation states of SIK2, I performed co-immunoprecipitation experiment with anti-Flag antibody using extracts from Flag-SIK2 and HA-PP1α cotransfected HEK293T cells (Fig4B, C).

To find out whether any putative PP1 docking motif exists in SIK2, I surveyed the SIK2 sequence for the consensus RVxF sequence. Indeed the sequence ^{16}RVGF^{19} matched the known PP1α docking motif (Fig.4A). To determine the ^{16}RVGF^{19}-dependent interaction between PP1α and SIK2, I constructed SIK2-F19Y, -V17A, -V17A/ F19Y and PP1α-C291R mutants. PP1α-C291R mutation could abolish its interaction with SIK2 (Fig.4B). The interaction between SIK2-F19Y, -V17A or -V17A/F19Y and PP1α remained unchanged (Fig.4C). However, the phosphorylation of SIK2 at T175 was elevated when SIK2-F19Y, -V17A or -V17A/F19Y was overexpressed (Fig. 4C). These results suggest that ^{16}RVGF^{19} is likely not the sole motif responsible for its interaction with PP1α.

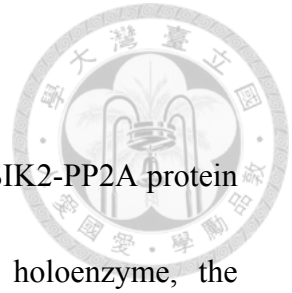
During the research on the interaction between SIK2 and PP1α, I became aware of a putative mutation on p97/VCP, ^{159}RAVEF^{163} ⇏ ^{159}RAVEG^{163}. That mutation is known to associate with ALS. To address the question of p97/VCP and PP1α

interaction, I conducted immunoprecipitation experiments for testing the interaction between wild-type or mutant p97/VCP and PP1α. Both wild-type and mutant p97/VCP could interact with PP1α. Similar to SIK2, only PP1α-C291R failed to interact with p97/VCP (Fig.4D). Together these results suggest that SIK2 and p97/VCP interact with PP1α through PP1α binding pocket, but may not the putative RVxF motif solely.

SIK2 regulates MEF2C-mediated gene expression

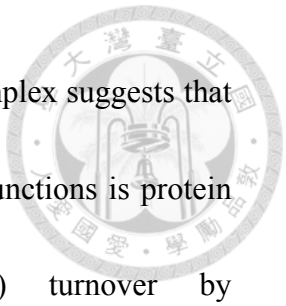
Previous results from our laboratory showed that depletion of SIK2 resulted in the activation of CaMKI and decreased PP2Ac level. PP2A was known to inactivate CaMK1 by dephosphorylating CaMKI-Thr177. Myocyte enhancer factor 2C (MEF2C) interacts with class II HDACs and bind DNA to repress the gene transcription. HDAC proteins are phosphorylated by CaMK (Ca^{2+}/calmodulin-dependent protein kinase) or other kinases, and translocated from the nucleus to cytoplasm [59,60]. To address the physiological function for SIK2-PP2A function, I performed the MEF2C-drived reporter assay. The transcription activity of MEF2C was increased when SIK2 level was reduced, even without overexpression of HDAC5 (Fig5A, B). These results suggest that when SIK2 is depleted, CaMK-mediated HDAC5 phosphorylation is responsible for the activation of MEF2C-mediated transcription. However, to rule-out

or rule-in the roles of SIK2 in this process, I performed overexpression of SIK2-WT, SIK2-KD and SIK2-S587A mutants. The results showed that overexpression of SIK2-WT resulted in activation of MEF2C (Fig5C, D) [61]. Taken together, these results suggest that SIK2 regulates MEF2C-mediated gene expression.



Discussion

One of the most intriguing findings from this study is that the SIK2-PP2A protein complex formation depends on PP2A holoenzyme. For PP2A holoenzyme, the catalytic C subunit is associated with the regulatory A subunit and serves as a scaffold for the recruitment of one of several B-type subunits. The B subunits determine the substrate specificity and intracellular localization of the PP2A holoenzymes [62]. Thus, the SIK2-PP2A complex may be responsible for targeting to specific subcellular localization and for dephosphorylating specific substrate. SIK2 is mainly an ER-resident kinase known to play regulatory role for autophagy when proteasome is inhibited [12]. *In vitro* enzymatic assay suggests that the phosphatase activity is preserved in the SIK2-PP2A complex (Lee et al., manuscript in preparation). This result seems to support that SIK2-PP2A complex could target to specific substrates. Another trivial question is that whether the kinase activity of SIK2-PP2A complex remains active. I have addressed this issue by immunoprecipitating overexpressed HA-PP2Ac and Flag-SIK2 with anti-HA antibody. The phosphorylated SIK2/T175 level is robustly maintained. SIK2 is activated by a two-step mechanism, an upstream kinase such as LKB1- or CaMKK-triggered autoactivation. When SIK2 is tethered to SIK2-PP2A complex, the two-step activation may be very efficient. The present results suggest that SIK2-PP2A complex contains both kinase and phosphatase activities. This



unprecedented union of two opposing activities in a two-protein-complex suggests that it may play unusual physiological functions. Among the possible functions is protein catabolism or intracellular organelle (e.g., mitochondria) turnover by ubiquitin-proteasome system (UPS) and autophagy. These processes are all associated with ER [63]. Depletion of SIK2 resulted in inhibiting UPS and autophagy while overexpression of SIK2 facilitated both processes [12]. The protein targets of phosphatase in SIK2-PP2A in UPS or autophagy are important future investigation goals.

Another important observation from this study is the hyper-phosphorylation of SIK2 when the cells were treated with higher concentration of OA (0.3 μM or higher), likely attributed to the inhibition of PP1. Lower concentration of OA (0.1 μM or lower) treatment did not result in significant hyper-phosphorylation of SIK2 indicates that PP2A is not involved in the dephosphorylation of SIK2. The negative regulation of SIK2 by PP1 is intriguing in that the kinase activity in SIK2-PP2A complex may be regulated by PP1 in a dynamic manner. OA treatment is very effective in disrupting SIK2-PP2A complex. Whether SIK2-PP2A complex is inaccessible to PP1 remains to be studied. Numerous PP1 regulatory proteins associate with PP1 via their anchoring RVxF motif. Both SIK2 and p97/VCP contain the consensus motif. My results indicated that PP1α can interact with SIK2 and p97/VCP, but the assembly may not

depend solely on their RVxF sequences. Additional PP1α regulatory subunits-dependent PP1α targeting to SIK2 or p97/VCP is possible. In addition, "SILK" and "MyPHoNE" motifs were also known as alternative docking motifs in some PP1 targets [30,64]. The docking site of PP1α is crucial for its associating with both SIK2 and p97/VCP indicating their association is likely through other unidentified sequences or indirectly through PP1α regulatory proteins.

Depletion of SIK2 results in enhanced activity of CaMKI as shown by elevated phosphorylated CaMKI/T177 level [29]. Phosphorylated CaMKI/T177 is a known target of PP2A. When SIK2 is depleted by shRNA-mediated knockdown, PP2A activity is decreased as evidenced by the decreased levels of PP2Ac and by the inhibition of PME-1. SIK2-PP2A complex cannot be accessed by PME-1 thus maintaining high phosphatase activity [29]. When SIK2 is overexpressed, the phosphorylated CaMKI/T177 level is decreased due to the phosphatase activity in the SIK2-PP2A complex. CaMKI has been shown to regulate MEF2-mediated gene expression by phosphorylating HDAC5, exporting it to the cytoplasm and relieving its binding to the promoter of MEF target genes [59,60]. Could SIK2-PP2A-mediated CaMKI inactivation or depletion of SIK2-induced CaMKI activation involve in MEF2-mediated gene expression? MEF2C participates in various cellular functions through gene regulation, such as muscle, blood vessel cardiac and neuron development

[60]. MEF2C inhibits gene expression by interacting with HDAC family protein, and enhanced gene transcription when HDAC proteins are phosphorylated and exported from nucleus.

Studies from our laboratory demonstrated CaMKI activity was regulated by SIK2 and PP2A [29]. My current results show that there are two pathways for SIK2 to regulate MEF2C-mediated transcription. SIK2 phosphorylates HDAC5 at S259 and direct up-regulate MEF2C gene expression. Knockdown of SIK2 decreased PP2A and increased MEF2C transcription through activation of CaMKI activity by decreased PP2A level (Lee et al., manuscript in preparation). The formation and regulation of SIK2-PP2A complex may influence other cellular program through CaMKI-mediated MEF2C regulatory system or yet to be uncovered mechanisms.

The phosphorylation of Snf1 at Thr210 had been demonstrated to regulate glycogen metabolism through PP1 and PP2A [65]. The interaction between SIK2, PP2A and PP1 suggests that the cross-talk between functional regulation of kinase and phosphatase. SIK2-PP2A complex may phosphorylate PP1 and reduce PP1 activity. PP1 influence SIK2 activity through feedback control. Another possible regulation between SIK2-PP2A complex and PP1 is that SIK2 phosphorylates the upstream kinase of PP1, such as inhibitor-1 at Thr35 [66]. These hypotheses remain to be proven.

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Figures

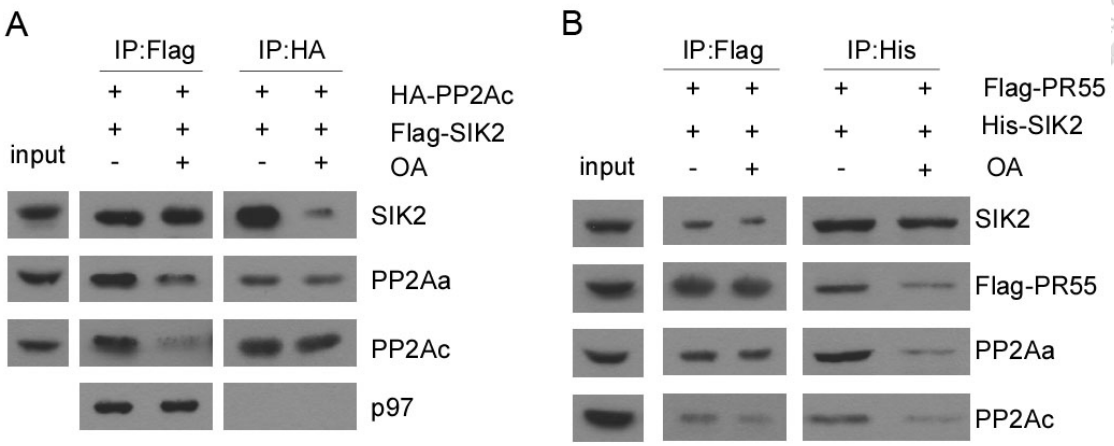


Figure 1. PP2A holoenzyme is required for its interaction with SIK2

(A) HEK293T cells were co-transfected with pCMV-Flag-SIK2 and pcDNA-HA-PP2Ac plasmids. Forty-eight hours after transfection, cells were prepared and immunoprecipitated with M2 (anti-Flag) or HA beads. The samples were analyzed by immunoblot analysis and probed with anti-SIK2 (15G10), anti-PP2Aa, anti-PP2Ac and anti-p97/VCP antibodies. Left panel: input control; middle panel: anti-Flag immunoprecipitates; right panel: anti-HA immunoprecipitates

(B) HEK293T cells were co-transfected with His-SIK2 and Flag-PR55 for Forty-eight hours. Cells lysates were prepared and immunoprecipitated with or without 1 μM okadaic acid by using M2 or Ni-NTA beads. The samples were analyzed by immunoblot analysis and probed with anti-SIK2 (15G10), anti-PP2Aa,

anti-PP2Ac and anti-Flag tag antibodies. Left panel: input control; middle panel: anti-Flag immunoprecipitates; right panel: Ni-NTA beads precipitates

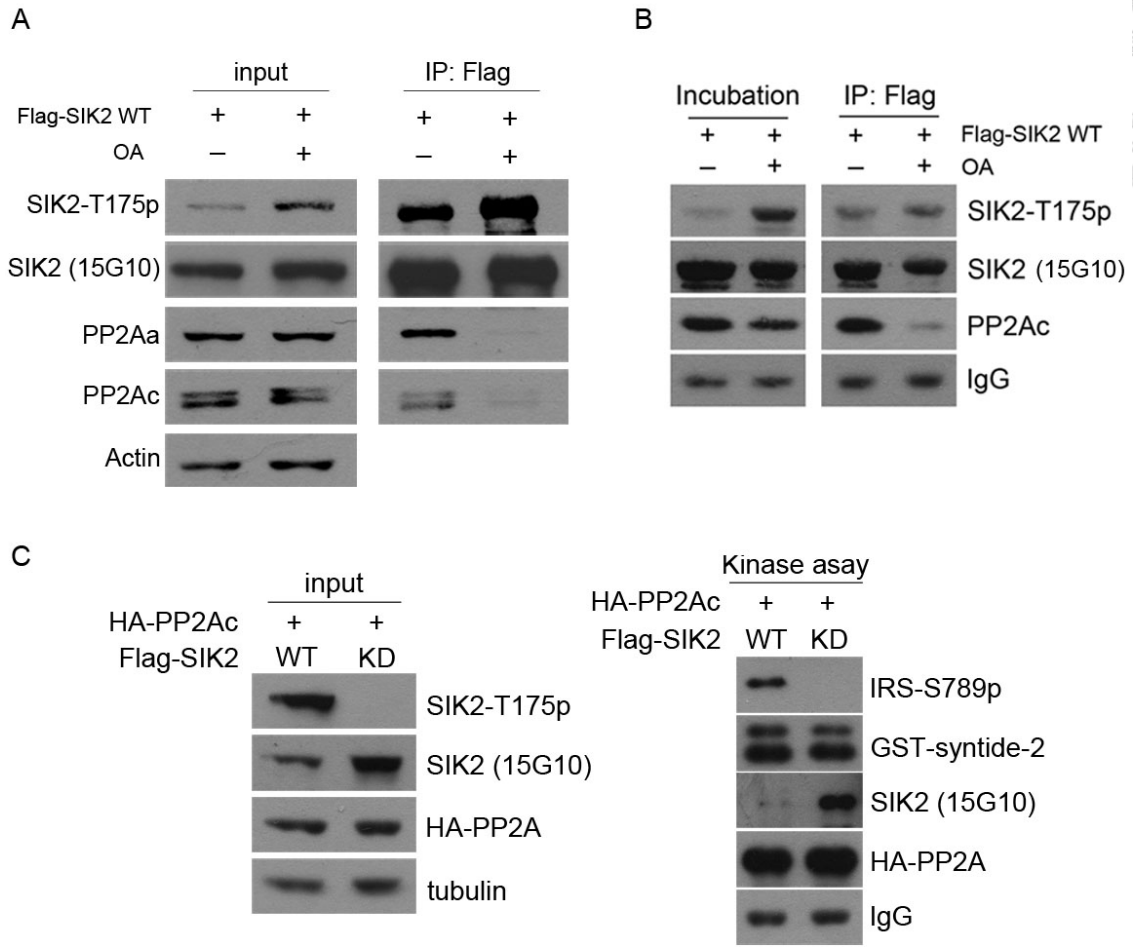


Figure 2. SIK2 kinase activity is preserved in SIK2-PP2A complex

(A) HEK293T cells were transfected with Flag-SIK2 plasmid. Twently-four hours after transfection, cells were treated with 0.1 μM okadaic acid or DMSO control for 4 hours, and then harvested with WCE lysis buffer. The cell extracts were immunoprecipitated with M2 beads in the presence or absence of 1 μM okadaic acid. Anti-SIK2-T175p antibody was used to detect the phosphorylation level of SIK2 at Thr175. Left panel: input control, right panel: anti-Flag immunoprecipitates

(B) The cell extracts prepared from Flag-SIK2 transfected HEK293T cells were

immunoprecipitated with M2 beads in the presence or absence of 1 μM OA. The immunoprecipitates were incubated in phosphatase assay buffer (20 mM Tris pH 7.5, 1 mM DTT, 5% glycerol, 10 mM $MgCl_2$, 2 mM EGTA and protease inhibitors) with or without OA at 30 °C for 30 minutes. Left panel: incubation at 30 °C, right panel: anti-Flag immunoprecipitates

(C) HEK293T cells expressing HA-PP2Ac, as well as wild-type Flag-SIK2 or kinase dead mutant Flag-SIK2 (KD). Forty-eight hours after transfection, cell extracts were prepared and immunoprecipitated with HA beads. The immune complex was subjected to kinase assay using purified GST-syntide-2 as substrate. Anti-IRS-S789p antibody was used to detect the phosphorylation level of syntide-2. Left panel: input control, right panel: kinase assay

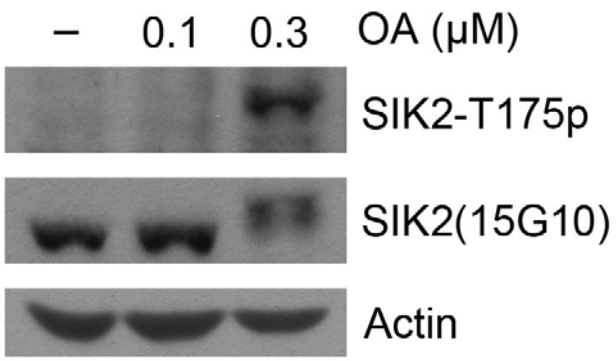
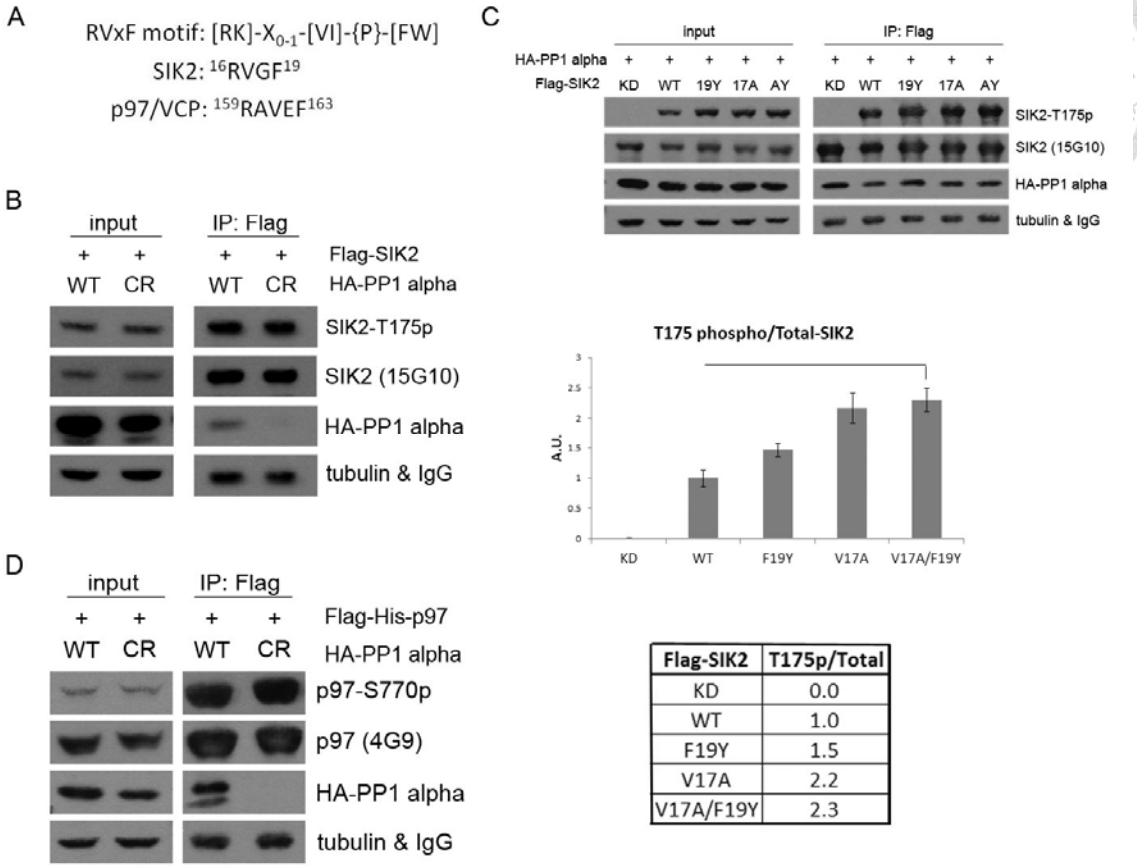


Figure3. High concentration of OA treatments resulted in elevated phosphorylation level of SIK2-T175

HEK293T cells were treated with 0.1 μM or 0.3 μM okadaic acid for 2 hours. Cell lysates were prepared with 6 M urea followed by immunoblot analysis with the anti-SIK2-T175p, anti-SIK2 (15G10) and α-tubulin (10D8) antibodies.



Flag-SIK2	T175p/Total
KD	0.0
WT	1.0
F19Y	1.5
V17A	2.2
V17A/F19Y	2.3

Figure 4. SIK2 and p97/VCP interact with PP1

(A) SIK2 and p97/VCP contain a consensus RVxF motif. (B) HEK293T cells were co-transfected with Flag-SIK2-WT, as well as HA-PP1α or HA-PP1α/C291R mutant plasmids. The cell lysates were immunoprecipitated with M2 beads. Left panel: input control; right panel: anti-Flag immunoprecipitates; CR: PP1α/C291R mutant (C) HEK293T cells were co-transfected with HA-PP1α-WT, as well as Flag-SIK2-WT, -KD, -F19Y, -V17A, or -F19Y/V17A. The cell lysates were immunoprecipitated with M2 beads. The histogram and table are quantification of western blot images (n=2)

and relative folds, respectively. Left panel: input control, right panel: anti-Flag immunoprecipitates, 19Y: F19Y mutant, 17A: V17A mutant, AY: V17A/F19Y double mutant (D) HEK293T cells were co-transfected with Flag-His-p97/VCP-WT, as well as HA-PP1α-WT or HA-PP1α/C291R plasmids as indicated. The cell lysates were immunoprecipitated with M2 beads. Anti-p97/VCP-S770p was used to detect the phosphorylation of p97/VCP at Ser770. Left panel: input control; right panel: anti-Flag immunoprecipitates

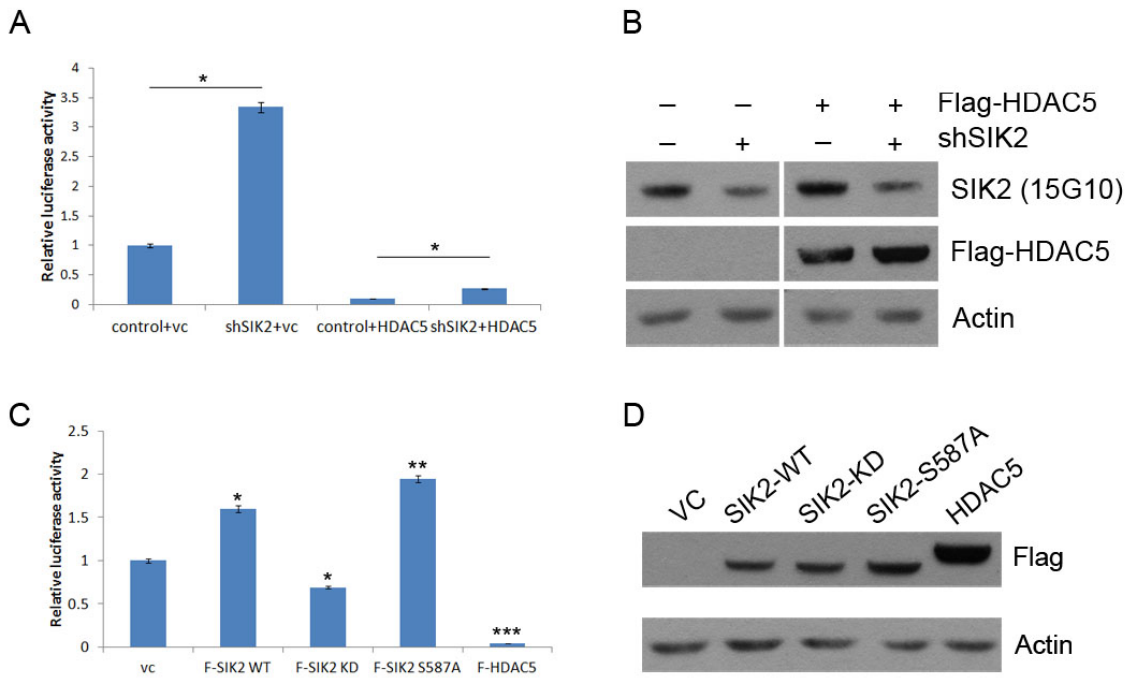


Figure 5. SIK2 regulates MEF2C-mediated transcription

(A) (B) HEK293T cells were infected with shSIK2 lentivirus. Forty-eight hours after infection, the cells were transfected with 3X-MEF2C-luciferase expressing reporter plasmid and pRL-TK vector. Cells also were co-transfected with pCMV-Taq2B vector or pCMV-Flag-HDAC5. Twenty-four hours later, cells were harvested and subjected to the reporter assay and immunoblot analysis. The reporter assay were represented triplicate experiments as the means ± S.D. *$p<0.05$ (C) (D) HEK293T cells were transfected with 3X-MEF2C-luciferase reporter plasmid and pRL-TK vector, and co-transfected with pCMV-Taq2B vector, Flag-SIK2-WT, -SIK2-KD, -SIK2-S587A or -HDAC5. Cells were harvested forty-eight hours post-transfection and subjected to the reporter assay and immunoblot analysis. The reporter assay were

represented as the means ± S.D.*p<0.05, **p<0.01 and ***p<0.001