Interaction between Salt-inducible Kinase 2 and Protein Phosphatase 2A Regulates the Activity of Calcium/Caldesmon-dependent Protein Kinase I and Protein Phosphatase Methylesterase-1*

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**Background:** SIK2 is the only AMPK family kinase that interacts with PP2A, with a hitherto unknown functional consequences.

**Results:** The interaction between SIK2 and PP2A preserves both enzyme activities and regulates CaMKI and PME-1 negatively.

**Conclusion:** The SIK2-PP2A complex is a critical regulator of calcium/caldesmon-mediated activation of CaMKI and PME-1.

**Significance:** SIK2-PP2A may play pivotal roles in regulating cell proliferation and stress response.

Salt-inducible kinase 2 (SIK2) is the only AMP-activated kinase (AMPK) family member known to interact with protein phosphatase 2A (PP2A). However, the functional aspects of this complex are largely unknown. Here we report that the SIK2-PP2A complex preserves both kinase and phosphatase activities. In this capacity, SIK2 attenuates the association of the PP2A repressor, the protein phosphatase methylesterase-1 (PME-1), thus preserving the methylation status of the PP2A catalytic subunit. Furthermore, the SIK2-PP2A holoenzyme complex dephosphorylates and inactivates Ca2+/calmodulin-dependent protein kinase I (CaMKI), an upstream kinase for phosphorylating PME-1/Ser15. The functionally antagonistic SIK2-PP2A and CaMKI and PME-1 networks thus constitute a negative feedback loop that modulates the phosphatase activity of PP2A. Depletion of SIK2 led to disruption of the SIK2-PP2A complex, activation of CaMKI, and downstream effects, including phosphorylation of HDAC5/Ser259, sequestration of HDAC5 in the cytoplasm, and activation of myocyte-specific enhancer factor 2C (MEF2C)-mediated gene expression. These results suggest that the SIK2-PP2A complex functions in the regulation of MEF2C-dependent transcription. Furthermore, this study suggests that the tightly linked regulatory loop comprised of the SIK2-PP2A and CaMKI and PME-1 networks may function in fine-tuning cell proliferation and stress response.

SIK2 is a serine/threonine protein kinase belonging to the AMP-activated protein kinase (AMPK) superfamily. AMPK

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2 The abbreviations used are: SIK2, salt-inducible kinase 2; AMPK, AMP-activated kinase; CREB, cAMP-response element-binding protein; ER, endoplasmic reticulum; VCP, valosin-containing protein; PP2A, protein phosphatase 2A; PME-1, protein phosphatase methylesterase-1; WCE, whole cell extract(s); CaMK, Ca++/calmodulin-dependent protein kinase; KD, kinase-dead; OA, okadaic acid.
SIK2-PP2A Regulates CaMKI and PME-1

subunits and a regulatory B subunit. The B subunit is responsible for the substrate specificity and subcellular localization. There are more than 20 different B subunits encoded by the human genome, and they can be grouped into four different families annotated as B/B55/PR55, B'/B56/PR61, B'/PR72, and B'/PR93/PR110, all of which share the same binding site on the core A subunit (11–13). Moreover, many of them undergo alternative splicing to generate different variants, further expanding the diversity of PP2A holoenzyme. Mechanisms governing the formation of heterotrimeric holoenzyme are important for maintaining its protein stability. Knockdown of either the A or C subunit accelerates the turnover of the other PP2A subunits in Drosophila S2 cells (17, 18). Additionally, mammalian PP2A C and most B subunits are stable only when they complex with the A subunit (19, 20). Some posttranslational modifications are known to influence PP2A holoenzyme formation or stability, such as phosphorylation of PP2Ac at Thr$^{308}$ and Tyr$^{307}$ (21, 22). In addition to regulation by phosphorylation, reversible methylation at the C-terminal leucine of the PP2Ac subunit provides another mechanism to regulate PP2A; carboxymethylation of Leu$^{609}$ was carried out by S-adenosylmethionine-dependent leucine carboxyl methyltransferase 1 and removed by PME-1 (23–25). Carboxymethylation of PP2Ac was shown to facilitate binding of the B/PR55 family (26, 27). The crystal structure of the PP2A holoenzyme showed a highly conserved C-terminal tail (3$^{$84}TPDYFL$^{309}$) of the C subunit that lies in the interface between the A subunit and B'/PR61γ, further linking its modification to B subunit recruitment (28).

PME-1 serves as a negative regulator of PP2A and can access only the A-C core and not the holoenzyme (29). The crystallographic structure of the PME-1-PP2Ac complex revealed that PME-1 mediates its inhibition partially by its demethylation activity on the PP2Ac C-terminal tail and by occupying the active site of PP2A (30). A catalytically inactive PP2Ac/H59Q mutant exhibits increased affinity for PME-1 and forms a stable complex in vivo (29). Furthermore, PME-1 gene disruption causes a perinatal lethality in mice (31). In glioma cells, PME-1 was shown to support ERK pathway signaling at a point upstream of Raf but downstream of PKC (32).

SIK2 is the only member of the AMPK family that can interact with PP2A (2); however, the functional impact of SIK2-PP2A interaction remains unknown. In this report, we showed that interaction between SIK2 and PP2A is important for preserving PP2A phosphatase activity by excluding the association of PME-1. We also discovered that there exists cross-regulation between CaMKI-PME-1 and SIK2-PP2A. The activity of CaMKI is inversely correlated to the level of SIK2-dependent PP2A activity (i.e. SIK2-PP2A complex). When the CaMKI activity is elevated, it phosphorylates PME-1 at Ser$^{115}$. Activated CaMKI negatively regulates SIK2, resulting in its degradation (8). Conversely, phosphorylated CaMKI/Thr$^{177}$ and PME-1/Ser$^{115}$ are substrates of PP2A. Both SIK2 and activated CaMKI could target HDAC5 for export to the cytoplasm and sequestration by 14-3-3, resulting in MEF2C-mediated transcription. Together, our present results suggest that cross-regulation between the SIK2-PP2A complex and CaMKI plays important physiological roles in coping with stress conditions or growth factor signaling cascades.

EXPERIMENTAL PROCEDURES

DNA Constructs and Antibodies—pCMV-FLAG-SIK2-WT, pCMV-FLAG-SIK2-KD (K49M), and pcDNA-His-SIK2-WT plasmids were cloned as described (9). Full-length CaMKI, PME-1, and PP2A B/PR55 cDNAs were cloned into mammalian expression vector pCMV-tag2B. HA-PP2Ac α1, CaMKI, and 14-3-3ɛ constructs were generated from RT-PCR and cloned into the pcDNA3.1-HA vector. A constitutively active CaMKI mutant was constructed by inserting cDNA (amino acids 1–294) of CaMKI into pCMV-tag vector. Dominant negative CaMKI (K49E) and PP2Ac/H59Q mutants were created by site-directed mutagenesis (Stratagene) according to the manufacturer’s instructions. The following primers were used for creating these mutants: CaMKI/K49E (5’-GCAGAAGCTGGTGCCATCGATGCATGGCAAGGAGG-3’) and PP2Ac/H59Q (5’-GGTACTGTCTGTGGAGATGTGCAGG-GCCAAATTCATGATCTC-3’). All mutants were verified by DNA sequencing. Two separate systems for SIK2 knockdown, shRNA directed at human SIK2 (5’-GCAGTGGTTGTATGAAACA-3’, NM_015191.1) was generated by using pSuper RNAi system (Oligoengine, Seattle, WA) and the plKO.1-shSIK2 (5’-GTCTGGATACAACATGATCATAT-3’; Clone ID TRCN0000037495 and TRCN0000037498) lentivirus was obtained from NRC (Academia Sinica, Taipei, Taiwan). For PP2A catalytic subunit knockdown, the shRNA targeting PP2Ac mRNA (5’-GAATTTCATGATCTC-3’) was generated by using pSuper RNai system. 3×MEF2-Luc reporter and pcDNA-MEF2C vector were kindly provided by Shen Liang Chen (National Central University, Taiwan).

Anti-PP2Ac (catalog no. 2259), PP2Aα (catalog no. 2309), and phosphospecific HDAC5/Ser$^{559}$ (catalog no. 3443) antibodies were purchased from Cell Signaling. Mouse monoclonal anti-methylated PP2Ac subunit (clone 2A10, catalog no. 04-1479) antibody was from Millipore. Rabbit anti-HA (A190-108A) antibody was from Bethyl Laboratories. The CaMKI/Thr$^{177}$ and PME-1/Ser$^{115}$ phosphospecific antibody were generated in rabbits by coupling synthetic phosphopeptides corresponding to the surrounding sequences of CaMKI/Thr$^{177}$ and PME-1/Ser$^{115}$ to KLH. Monoclonal antibodies against human SIK2 (clone 15G10), β-tubulin (clone 10D8), PME-1 (clone 20G3), and p97/VCP (clone 4G9) as well as rabbit-derived anti-FLAG and anti-α-actin antibodies were generated in our laboratory (9).

Cell Culture and Transfection—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Biological Industries) and 100 units/ml penicillin and streptomycin (Invitrogen) at 37 °C in a 5% CO$_2$ humidified incubator. TurboFectTM (Fermentas) transfection was carried out according to the manufacturer’s instructions.

Immunoprecipitation and Western Blotting—Whole cell extracts (WCE) were prepared by lysing the cells in WCE buffer (0.2 M NaCl, 20 mM Tris–HCl, pH 7.5, and 0.1% Triton X-100) supplemented with a mixture of protease and phosphatase.
SIK2•PP2A Regulates CaMKI and PME-1

inhibitors (0.5 mM NaF, 1 mM leupeptin, 1 mM pepstatin A, 1 mM PMSF, 1 mM sodium butyrate, and 1 mM sodium orthovanadate) on ice for 20 min. WCE was incubated with M2 beads (Sigma) for 1 h at 4 °C. After incubation, the immunoprecipitates were washed three times in WCE buffer, added SDS-PAGE sample buffer (30 mM Tris-HCl, pH 6.8, 3% SDS, 0.015% bromphenol blue, 1.5 mM urea, and 8% glycerol), and separated by SDS-PAGE. After the electrophoresis, the proteins were transferred to PVDF membrane and blocked for 30 min in blocking solution (5% nonfat milk in PBS-T) at room temperature. The membrane was probed with antibody for 1 h at room temperature, washed three times in PBS-T (PBS containing 0.1% Tween 20). The membrane was incubated with an HRP-labeled secondary antibody and washed three times in PBS-T. The immunoblot was detected with enhanced chemiluminescence (PerkinElmer Life Sciences).

Fractionation of SIK2-containing Complex by Superdex 200 FPLC—FLAG-SIK2-containing whole cell extracts (0.2 ml/1 mg) were subjected to Superdex 200 column (1.4 × 40 cm) FPLC. A fraction of 0.5 ml was collected and precipitated with 5% TCA, rinsed with acetone, dried, and dissolved in SDS sample buffer for Western blot analysis.

Protein Purification and in Vitro Phosphatase and Kinase Assay—FLAG-tagged CaMKI and PME-1 were immunoprecipitated with M2 beads and washed with high salt and detergent buffer (TNT: 20 mM Tris-HCl pH 7.5, 0.5 mM NaCl, and 0.5% Triton X-100) supplemented with 50 mM NaF and 20 μM okadaic acid, to remove the associated phosphatases and other interacting proteins. Recombinant substrates were eluted by the addition of 2 μg of FLAG peptide in 20-μl reaction mixtures and incubated for 30 min at room temperature. The amounts and purity of substrate was verified by Coomassie Blue staining of SDS gel.

Phosphatase activity assay was performed following the protocol described below. Wild type PP2A and catalytically inactive (H59Q) mutant were immunoprecipitated with anti-HA beads. Washed beads were resuspended in assay buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM EGTA, 10 mM MgCl2, and 0.1 mg/ml BSA) and incubated with substrate at 30 °C for 30 min. The reaction was terminated by the addition of SDS sample buffer and subjected to immunoblot analysis. An assay for SIK2-associated PP2A activity was performed similarly; FLAG-SIK2-WT and FLAG-SIK2-KD mutant-associated PP2A were immunoprecipitated by using M2 beads instead. SIK2•PP2A complex was prepared by immunoprecipitating the HA-tagged PP2A-associated SIK2 with anti-HA beads, and an SIK2 kinase assay was performed as described previously (9).

FLAG-tagged PME-1 was immunoprecipitated with M2 beads and washed with TNT buffer. To elute the substrate, 2 μg of FLAG peptide was added to the 30-μl immunoprecipitates and incubated for 30 min at room temperature. The purity of the substrate was verified by Coomassie Blue staining of SDS gel. Recombinant FLAG-CaMKI was also purified similarly. CaMKI was preactivated with 10 mM CaCl2 during lysis of cells. The CaMKI was used for a kinase assay immediately after immunoprecipitation.

For the CaMKI kinase assay, wild type CaMKI and dominant negative (K49E) mutant were assayed at 30 °C by incubation in the following reaction mixtures: 50 mM Tris-Cl, pH 7.5, 1 mM CaCl2, 0.5 mM DTT, 0.5 mg/ml BSA, 0.2 mM ATP, and 1 μg of PME-1 substrate. The reaction was terminated by SDS-sample buffer and subjected to Western blot analysis.

**RESULTS**

Complex Formation between SIK2 and PP2A—SIK2 is the only member of the AMPK family that can interact with PP2A (33). Our previous results have shown that interaction between SIK2 and p97/VCP regulates ER-associated protein degradation (10). Interestingly, when PP2Ac was overexpressed, the p97/VCP level in both wild type SIK2 (WT) and kinase-dead mutant SIK2 (KD) immunoprecipitates was reduced, implying that PP2A and p97/VCP may exist in two distinct SIK2-containing complexes (Fig. 1A). To further confirm this possibility, the ectopically expressed SIK2 from HEK293T lysates was subjected to Superdex-200 FPLC fractionation. Differential fractionation of p97/VCP and PP2A (with elution peaks at fractions 25 and 31, respectively; Fig. 1B) suggests that PP2A and p97/VCP form distinct complexes with SIK2. Additionally, an immunoprecipitation assay showed that the interaction between SIK2 and PP2A did not require the kinase activity of SIK2 because both SIK2-WT and SIK2-KD could immunoprecipitate comparable levels of PP2Ac (Fig. 1A).

To determine which subunit of the heterotrimeric PP2A is responsible for interaction with SIK2, we tested whether catalytically inactive PP2Ac/H59Q mutant could interact with SIK2. H59Q mutant reportedly associates with adaptor A subunit but is unable to recruit B subunit (29). Such mutation also led to the loss of SIK2•PP2Ac interaction (Fig. 1C). To further test whether SIK2 interacts with PP2A in a phosphatase activity-dependent manner, extracts were prepared from HEK293T cells transfected with epitope-tagged SIK2 and PP2Ac in the presence of okadaic acid and immunoprecipitated with epitope-specific antibodies. The results showed that okadaic acid treatment disrupted the complex formation between SIK2 and PP2A (Fig. 1D). Next, to confirm the interaction between SIK2 and PP2A holoenzyme, we overexpressed SIK2 and the regulatory subunit B/PR55 in HEK293T cells and performed immunoprecipitation. We subsequently observed that SIK2 and B/PR55-containing PP2A holoenzyme co-existed in the same immunocomplex but lost such
association in the presence of okadaic acid (Fig. 1E). On the contrary, SIK2 and p97/VCP complex formation was not affected by okadaic acid (Fig. 1, D and E). Together, these results indicate that SIK2 interacts with the catalytically active PP2A holoenzyme.

The Phosphatase Activity Is Preserved in SIK2-PP2A Complex by Excluding PME-1 from Associating with PP2A—To address the functional consequence of the SIK2-PP2A interaction, we performed further biochemical experiments for the SIK2-PP2A complex. When the level of SIK2 was reduced by shRNA-mediated knockdown, the levels of total and carboxymethylated PP2Ac also dropped (Fig. 2A). However, in cells with overexpressed SIK2-WT or SIK2-KD, the levels of PP2Ac and carboxymethylated PP2Ac remained unchanged (Fig. 2B). Because carboxymethylation of PP2Ac/Leu<sup>309</sup> facilitates PP2A holoenzyme formation and is a hallmark of active PP2A (26), these results suggest that PP2A expression and activity may be preserved in the SIK2-PP2A complex. Further comparison of the PP2Ac/L309Me levels from total and SIK2-immunoprecipitated PP2Ac revealed that SIK2-associated PP2Ac was enriched in the methylated form (Fig. 2C). These results thus suggest that the SIK2-PP2A complex could protect PP2A from the endogenous inhibitor, PME-1. To clarify the relationship between SIK2-PP2A complex and PME-1, the

FIGURE 1. Holoenzyme of PP2A is required for SIK2 interaction. A, HEK293T cells were transfected with FLAG-SIK2-WT or SIK2-KD and HA-PP2Ac for 48 h. Cell lysates were probed with anti-FLAG, p97/VCP, PP2Ac, or HA antibodies (left). Anti-FLAG immunoprecipitates (IP) were probed by Western blot with the same antibodies as used in the left panel (right). B, 48 h after transfection with FLAG-SIK2-WT, HEK293T cells were harvested and lysed in WCE buffer, followed by FPLC fractionation to separate protein complex by its mass. Each protein fraction was precipitated in 5% TCA and dissolved in SDS sample buffer for Western blot analysis. C, HEK293T cells were transfected with FLAG-SIK2-WT or FLAG-SIK2-KD and HA-PP2Ac-WT or HA-PP2Ac/H59Q for 48 h. Cell lysates were immunoprecipitated (IP) by anti-FLAG or anti-HA beads and subjected to immunoblotting analysis. D, HEK293T cells were transfected with FLAG-SIK2-WT and HA-PP2Ac-WT for 48 h. Cells lysates were immunoprecipitated by anti-FLAG or anti-HA beads. During the immunoprecipitation, 20 μM OA was added to interrupt the PP2Ac holoenzyme formation and then subjected to immunoblotting analysis. E, HEK293T cells were transfected with His-SIK2-WT and FLAG-PR55 for 48 h. Cell lysates were immunoprecipitated by anti-FLAG or nickel beads with 20 μM OA and then subjected to immunoblotting analysis.
SIK2-PP2A Regulates CaMKI and PME-1

endogenous inhibitor of PP2A, we performed immunoprecipitation experiments on HEK293T cell extracts co-expressing FLAG-SIK2 and HA-PP2Ac. Our data showed that PME-1 was excluded from the SIK2/PP2A complex, whereas PP2A and PME-1 formed a distinct complex (Fig. 2D). These results thus suggest that the SIK2-PP2A could protect PP2A activity from the inhibition of PME-1.

Furthermore, we observed an up-regulation of activated phosphorylation of SIK2/Thr175 level as well as down-regulation of repressive phosphorylation of SIK2/Ser587 in the complex (Fig. 2E), which may indicate that SIK2 activity is also preserved in association with PP2A. To further confirm the kinase activity in the SIK2-PP2A complex, we performed co-transfection of HEK293T cells with SIK2-WT or SIK2-KD with HA-PP2Ac. SIK2-PP2A complex was prepared by immunoprecipitation of HA-PP2Ac and then assayed for kinase activity with GST-Syntide substrate. The results showed that the SIK2-PP2A complex is indeed active in phosphorylating this substrate (Fig. 2F). Taken together, these results imply that the PP2A holoenzyme-dependent SIK2-PP2A complex formation has the distinct function of preserving both SIK2 and PP2A activities.

Negative Regulation of CaMKI by PP2A—As a known substrate of PP2A, CaMKIV can be inactivated and dephosphorylated in vitro by PP2A (35). Characterization of phosphospecific antibody was shown in Fig. 3A. To address whether the functionally related CaMKI is negatively regulated, we next investigated whether PP2A is also involved in its inactivation. To this end, we found that when PP2A activity was inhibited by okadaic acid or under overexpression of PP2Ac/H59Q, the phosphorylated CaMKI/Thr177 level increased (Fig. 3, B and C). Similarly, when PP2Ac was down-regulated by shRNA, the phosphorylated CaMKI/Thr177 level was elevated (Fig. 3D). In vitro phosphatase assay using immunoprecipitated HA-tagged PP2A complex further demonstrated that CaMKI/Thr177 is specifically dephosphorylated by PP2A (Fig. 3E). These findings there-

FIGURE 2. Depletion of SIK2 resulted in reduced levels of both PP2Ac and carboxymethylated PP2Ac (PP2Ac/L309Me). Western blot analysis of endogenous PP2Ac and carboxymethylated PP2Ac in HEK293T cells transfected with pSuper-shLuc/shSIK2 plasmids (A) or FLAG-SIK2-WT/KD (B) for 48 h. The ratio of quantitative results is expressed as means ± S.D. (error bars) of three independent experimental (*, p < 0.05; N.S., not significant). C, HEK293T cells were transiently transfected with FLAG-SIK2 for 48 h, and then cell extracts were immunoprecipitated (IP) with M2 beads. The carboxymethylated PP2Ac in the immunocomplexes was analyzed by Western blotting. D, HEK293T cells were transfected with FLAG-SIK2 and HA-PP2Ac for 48 h. Cell lysates were immunoprecipitated by immobilized anti-FLAG or anti-HA antibodies and then subjected to immunoblotting analysis. E, immunoblot analysis of phosphorylated SIK2/Thr175 and SIK2/Ser587 in HA-tagged PP2A immunoprecipitates is shown on the left. All quantitative values were calculated as phosphorylated SIK2 (e.g. SIK2/Thr175 or SIK2/Ser587) normalized with total SIK2 level and plotted as a bar graph in the right panel. Quantitative data are means ± S.D. of three experimental replicates (***, p < 0.001). F, HEK293T cells were co-transfected with FLAG-SIK2-WT or FLAG-SIK2-KD and HA-PP2Ac. SIK2-PP2A complex was prepared by immunoprecipitation of HA-PP2Ac. The kinase activity was assayed with GST-Syntide substrate.

21112 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 289 • NUMBER 30 • JULY 25, 2014
fore indicate that PP2A is responsible for the dephosphorylation of CaMKI/Thr177.

Cross-regulation between SIK2/PP2A Complex and CaMKI—CaMKI is known to negatively regulate SIK2 through Thr484 phosphorylation, which is linked to its degradation (8). Overexpression of constitutively active CaMKI indeed led to a reduction in the SIK2 protein level in HEK293T cells (Fig. 4A). To address whether there exists a cross-regulation between CaMKI and SIK2, we performed SIK2 knockdown along with CaMKI overexpression in HEK293T cells. Immunoblotting analysis showed that SIK2 knockdown enhanced the phosphorylation level of CaMKI/Thr177 (Fig. 4B). However, there was no difference in this phosphorylation level upon SIK2-WT or SIK2-KD overexpression (Fig. 4C), suggesting that depletion of SIK2 resulted in CaMKI activation. To further elucidate how SIK2 may affect the activity of CaMKI, we focused on the functional characterization of SIK2 and PP2A complex. In vitro phosphatase activity of immunoprecipitated SIK2/PP2A complex demonstrated that the SIK2/PP2A complex serves as a negative regulator for CaMKI by dephosphorylation of CaMKI/Thr177 (Fig. 4D). Taken together, these data suggest that depletion of SIK2, with the consequence of SIK2/PP2A complex disruption, contributes to CaMKI activation.

CaMKI Is the Upstream Kinase for Phosphorylation of PME-1/Ser15—We have established that down-regulation of SIK2 resulted in decrease of PP2Ac and PP2Ac/L309Me levels (Fig. 2A). We further noticed that the amino acid sequence surrounding the PME-1/Ser15 bears similarity to the consensus target of AMPK, CaMKI/IV, SIK1, or SIK2 (10LGRLpSR-PP1/9) (Fig. 5A). However, the increased phosphorylated PME-1/Ser15 level in response to SIK2 knockdown excluded the possibility that it is a target of SIK2 (Fig. 5, B and C). Besides, the phosphorylated PME-1/Ser15 level was also elevated under oka-daic acid treatment (Fig. 5D). Before substrate purification, FLAG-CaMKI-expressing HEK293T cells were pretreated with 100 nM OA for 2 h to enrich the phosphorylated CaMKI/Thr177 level. Anti-HA immunoprecipitates were mixed with purified FLAG-CaMKI in 30 μl of assay buffer. The reaction mixture was incubated at 30 °C for 30 min. The reaction was terminated by SDS sample buffer and subjected to Western blot analysis (top). The expression of these recombinant proteins in the lysate input was probed with anti-FLAG and anti-HA antibodies (bottom). The purity of substrate, FLAG-CaMKI, was examined by Coomassie Blue (CB) staining (top right). Quantitative data of B–D are expressed as means ± S.D. of three independent experiments (*, p < 0.05; **, p < 0.005).
SIK2-PP2A Regulates CaMKI and PME-1

cate that the elevated phosphorylation levels of both CaMKI/Thr177 and PME-1/Ser15 are inversely correlated with the PP2A activity associated with SIK2-PP2A complex. Next, to substantiate whether PME-1/Ser15 is reciprocally targeted by CaMKI-mediated phosphorylation, we performed an in vitro kinase assay using puriﬁed recombinant CaMKI and PME-1. Our results indeed showed that PME-1 could be phosphorylated in vitro by active CaMKI (Fig. 6A). In addition, this phosphorylation could be suppressed by the addition of the CaMKI inhibitor, KN-93 (Fig. 6B), but elevated by overexpression of the constitutively active CaMKI (Fig. 6C). Collectively, these data are in line with the scenario that PME-1 serves as downstream regulator of SIK2-PP2A-CaMKI signaling, and CaMKI is the upstream kinase responsible for PME-1/Ser15 phosphorylation.

HDAC5-mediated Transcriptional Inhibition of MEF2C Is Regulated by SIK2-PP2A—Previous studies demonstrated that CaMK family kinases contribute to MEF-dependent transcriptional regulation (36). In this capacity, class IIa HDACs (e.g. HDAC4 and -5) are phosphorylated by CaMK and sequestered to the cytoplasm by binding to 14–3–3 (37). To investigate whether the SIK2-PP2A-CaMKI signal cascade participates in modulation of MEF-dependent transcription, we probed the phosphorylation of HDAC5/Ser259 in the context of SIK2 knockdown. Our results showed that depletion of SIK2 led to increased phosphorylation level of HDAC5/Ser259 (Fig. 7A). Immunofluorescence staining also revealed a greater extent of HDAC5 cytoplasmic localization in the knockdown cells (Fig. 7B). Consistent with these changes, a co-immunoprecipitation experiment showed increased association of 14–3–3 with HDAC5 when SIK2 was depleted (Fig. 7C). To address the functional consequence of such regulation in a transcriptional context, a 3×MEF binding site-containing promoter reporter assay was performed. The reporter activity was induced by depletion of SIK2 (Fig. 7D). Previous studies also showed that HDAC5/Ser259 could be phosphorylated by AMPK and SIK1 (38–40), and that SIK2 may also target HDAC5/Ser259. Under SIK2 overexpression, the MEF2C-mediated transcription was activated comparably with that of SIK2 depletion (Fig. 7E). These results therefore suggest that SIK2 can phosphorylate HDAC5/Ser259 and trigger its sequestration in the cytoplasm, a function similar to depletion of SIK2-activated CaMKI.

Because the kinase activity is preserved in the SIK2-PP2A complex (Fig. 2E), we addressed whether the SIK2 in that complex could phosphorylate HDAC5/Ser259. Overexpression of SIK2-PTT seems to have a dominant effect over the presence of recombinant PP2A-PTT or PP2A/H59Q mutant (Fig. 7F) on the phosphorylation of HDAC5/Ser259. These results suggest that SIK2 (as shown by the absence of complex formation by SIK2-PP2A/H59Q) per se or in the context of the SIK2-PP2A-PTT complex is responsible for HDAC5/Ser259 phosphorylation, and activation of MEF2C-dependent transcrption (Fig. 8).

DISCUSSION

This study reported the interaction between SIK2 and PP2A that preserves the kinase and phosphatase activities of SIK2 and PP2A, respectively. These findings extended our previous results that demonstrated a link of SIK2 to p97/VCP and the process of ER-associated protein degradation (2) and thus broadened the functional implications of SIK2 in cells. Our present results further showed that the PP2A activity is preserved in the SIK2-PP2A complex and serves as a negative regulator for the activation of the CaMKI and PME-1 network.
Interestingly, knockdown of SIK2 not only caused disruption of SIK2/PP2A complex but also resulted in elevation of the Bcl-2 level in the ER membrane (data not shown). This may represent an alternative mechanism through which SIK2 regulates CaMKI-associated signaling; when the Bcl-2 level is elevated in the ER, Ca\(^{2+}\) may be released and consequently activates CaMKI (i.e., elevation of CaMKI/Thr\(^{177}\) phosphorylation level) (41). Furthermore, CaMKI-mediated phosphorylation of SIK2/Thr\(^{484}\) is known to down-regulate SIK2 through protein degradation (8). Indeed, the SIK2 level was reduced by overexpressing constitutively active CaMKI (Fig. 4A). These findings are thus in line with the negative feedback regulatory mechanism between these two kinases. In this scenario, in addition to stabilizing and activating PP2A, formation of the SIK2/PP2A complex and the consequently elevated phosphatase activity may also block CaMKI-mediated phosphorylation and degradation of SIK2. Together, these results highlight a tightly linked regulatory loop composed of SIK2/PP2A, CaMKI, and PME-1 that may function antagonistically in fine-tuning cell proliferation and stress response.

The N-terminal sequence of PME-1 is an unstructured, flexible region that may have hitherto unknown regulatory functions. Our present results showed that PME-1/Ser\(^{15}\) is phosphorylated by CaMKI and dephosphorylated by PP2A, suggesting that the phosphorylation/dephosphorylation of PME-1/Ser\(^{15}\) may have important regulatory functions. Our
results also demonstrated that the phosphorylated levels of PME-1/Ser\textsuperscript{15} and CaMKI/Thr\textsuperscript{177} are inversely correlated with the phosphatase activity of SIK2-PP2A complex, further implying that the demethylase activity of phosphorylated PME-1/Ser\textsuperscript{15} may be higher than that of its unphosphorylated state. Furthermore, our findings on the SIK2-PP2A complex and their relationship with PME-1 may provide important insights into several human diseases and pathophysiological conditions. Given that PP2A is a major Tau phosphatase, its activity may also contribute to the development of Alzheimer disease. In addition, PP2A is also known to function as a tumor suppressor (42, 43), whereas PME-1 is a repressor of PP2A that enhances oncogenic signaling (32). Aza-H9252-lactam, an inhibitor of PME-1, is a known powerful drug for inhibiting cancer cell growth (44). When the catalytic center is bound by aza-H9252-lactam, PME-1 can no longer inhibit PP2A, whose activity may thus be preserved for attenuating the oncogenic signaling pathways. Therefore, based on the observation that SIK2-depleted cancer cells are susceptible to apoptosis (7), we propose that inhibitor of PME-1, a number of endogenous and viral inhibitors of PP2A have been studied (16, 45). Whether the SIK2-PP2A complex could protect the inhibition of PP2A by one or more of these inhibitors remains unclear.

Evidence supporting interactions between SIK2 and PP2A includes the following: 1) the lack of interaction between the catalytically inactive H59Q mutant of the C subunit, which is defective in holoenzyme formation (29), and SIK2, and 2) the association of SIK2 with recombinant PR55-containing PP2A\textsubscript{a} and PP2A\textsubscript{c}. These data clearly suggest that the PP2A holoenzyme structure is important for SIK2-PP2A complex formation. However, the actual physical interaction between SIK2 and PP2A remains to be elucidated by structural studies. The outcome of these future investigations may shed light on how to manipulate PP2A activity in SIK2-PP2A complex formation. Intriguingly, regulatory parallelism between SIK2-PP2A and immunoglobulin-binding protein 1 (IGBP1/\textalpha4)-PP2A complexes was noticed. The IGBP1-PP2A complex preserves PP2A activity and functions in microtubule dynamics and mTORC1 signaling (46, 47). The Opitz syndrome protein midline 1 (MID1) serves as a microtubule targeting subunit as well as a negative regulator of IGBP1-PP2A. MID1 has been shown to function as an E3 ligase targeting the PP2Ac for ubiquitin-mediated degradation (48,
49). Depletion of MID1 or expression of Opitz syndrome-derived mutated MID1 was shown to compromise association with microtubules and/or transport along microtubules. In an analogous manner, CaMKI is a negative regulator of phosphorylating SIK2/Thr484 and targeting it for proteasomal degradation. Thus, activation of CaMKI by abnormal elevation of calcium may trigger the disruption of the SIK2/PP2A complex, exposing PP2A to its endogenous inhibitor, such as PME-1. The effects of decreased SIK2/PP2A complex may thus be linked to the dysregulation of microtubule vesicle transport and have great impacts on the functions of SIK2 in autophagy and ER-associated protein degradation, as exemplified by our previous publications (9, 10). The physiological functions of SIK2/PP2A complex are important questions to be addressed in future research.

SIK1, a closely related kinase of SIK2, exhibits a seemingly distinct role in the regulation of the CaMKI and PME-1 pathway. In response to elevated intracellular calcium, CaMKI is activated to phosphorylate SIK1 on Thr322, which in turn is responsible for the phosphorylation and activation of PME-1. Consequently, PME-1 dissociates from PP2A and exerts activation of the Na+/H+-ATPase (34). Contrary to this regulatory
SIK2-PP2A regulates CaMKI and PME-1

Mechanism, our results showed that CaMKI directs phosphorylation of PME-1/Ser15 and that the SIK2 level is inversely correlated with the phosphorylation states of CaMKI/Thr777 and PME-1/Ser15. Together, these findings emphasize distinct roles of these related kinases that closely depend on cellular contexts.

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