Phosphorylation of the CREB-specific coactivator TORC2 at Ser\(^{307}\) regulates its intracellular localization in COS-7 cells and in the mouse liver

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Uebi T, Tamura M, Horike N, Hashimoto YK, Takemori H. Phosphorylation of the CREB-specific coactivator TORC2 at Ser\(^{307}\) regulates its intracellular localization in COS-7 cells and in the mouse liver. Am J Physiol Endocrinol Metab 299: E413–E425, 2010. First published June 15, 2010; doi:10.1152/ajpendo.00525.2009.—The CREB-specific coactivator TORC2 (also known as CRTC2) upregulates gluconeogenic gene expression in the liver. Salt-inducible kinase (SIK) family enzymes inactivate TORC2 through phosphorylation and localize it in the cytoplasm. Ser\(^{171}\) and Ser\(^{275}\) were found to be phosphorylated in pancreatic \(\beta\)-cells. Calcineurin (Cn) is proposed as the Ser\(^{275}\) phosphatase, because its inhibitor cyclosporin A (CsA) stabilizes phospho-Ser\(^{275}\) and retains TORC2 in the cytoplasm. Because the regulation of dephosphorylation at Ser\(^{171}\) has not been fully clarified, we performed experiments with a range of doses of okadaic acid (OA), an inhibitor of PP2A/PPI, and with overexpression of various phosphatases and found that PP1 functions as an activator for TORC2, whereas PP2A acts as an inhibitor. In further studies using TORC2 mutants, we detected a disassociation between the intracellular distribution and the transcription activity of TORC2. Additional mutant analyses suggested the presence of a third phosphorylation site, Ser\(^{307}\). The Ser\(^{307}\)-disrupted TORC2 was constitutively localized in the nucleus, but its coactivator activity was normally suppressed by SIK1 in COS-7 cells. CsA, but not OA, stabilized the phosphogroup at Ser\(^{307}\), suggesting that differential dephosphorylation at Ser\(^{171}\) and Ser\(^{307}\) cooperatively regulate TORC2 activity and that the nuclear localisation of TORC2 is insufficient to function as a coactivator. Because the COS-7 cell line may not possess signaling cascades for gluconeogenic programs, we next examined the importance of Ser\(^{307}\) and Ser\(^{171}\) for TORC2’s function in mouse liver. Levels of phosphorylation at Ser\(^{307}\) and Ser\(^{307}\) changed in response to fasting or fed conditions and insulin resistance of the mouse liver, which were modified by treatment with CsA/OA and by overexpression of PP1/PP2A/Cn. These results suggest that multiple phosphorylation sites and their phosphatases may play important roles in regulating TORC2/CREB-mediated gluconeogenic programs in the liver.

cAMP response element-binding protein; transducer of regulated cAMP response element-binding protein activity 2; nuclear export; salt-inducible kinase

THE CAMP RESPONSE ELEMENT (CRE)-binding protein (CREB) performs numerous physiological functions in response to a variety of stimuli and stresses (14, 17, 29, 34). Expression of genes involved in gluconeogenesis in the liver, such as the peroxisome proliferator-activated receptor-\(\gamma\) coactivator-1\(\alpha\) (PGC-1\(\alpha\)) and phosphoenolpyruvate carboxykinase (PEPCK) genes, is also regulated by CREB, which is activated by glucagon under fasting conditions (12, 45). Glucagon increases the level of cellular cAMP followed by activation of protein kinase A (PKA), resulting in enhanced activation of phosphorylation of CREB at Ser\(^{133}\) (37, 44). Phospho-CREB then initiates transcription by recruiting the coactivator CREB-binding protein (CBP)/p300 (4, 24).

To regulate the gluconeogenic program, insulin counters the action of cAMP signaling (32). Paradoxically, insulin signaling is also able to phosphorylate Ser\(^{133}\) via the Ras-MAPK or PKC pathway (21), whereas the level of phospho-CREB at Ser\(^{133}\) does not change in the liver of mice treated with insulin (22). The other type of coactivator, transducer of regulated CREB activity 2 (TORC2), also called CRT2 (5, 15), is expressed in the liver and shuttles between the nucleus and the cytoplasm in response to glucagon/cAMP signaling. Formation of the CREB-TORC2 complex requires the bZIP domain of CREB and the coiled-coil region of TORC2 but not phosphorylation of CREB at Ser\(^{133}\) (22).

Under nonstimulating conditions, TORC2 is highly phosphorylated and is localized in the cytoplasm (2, 35). Once cAMP-PKA signaling is initiated, TORC phosphatase dephosphorylates TORC2, and this dephospho-TORC2 migrates into the nucleus, binds to CREB, and activates CREB-mediated transcription. Ser\(^{171}\) has been identified as the critical site of phospho/depshorylgroup of TORC2 (20, 35).

Depletion of TORC2 in hepatocytes using RNAi techniques results in a weakened response to glucagon/cAMP signaling and hypoglycemia (11, 22). Similar metabolic modifications of CREB-mediated gluconeogenesis also result from overexpression of TORC kinases, such as salt-inducible kinase (SIK) (7, 20). Compounds such as 5-aminoimidazole-4-carboxamidine-1-\(\beta\)-d-ribofuranoside (AICAR) and metformin, which activate AMP-activated protein kinase (AMPK), increase TORC2 phosphorylation and suppress CREB-mediated gluconeogenesis (22). In contrast, RNAi used against SIK1/SIK2 or a small compound that potently inhibits SIK activity, such as staurosporine, accelerates dephosphorylation of TORC2 (19). In addition to the above inactivation of SIKs, PKA inhibits the TORC kinase activity of SIKs through phosphorylation at Ser\(^{307}\) (SIK1) (41), Ser\(^{307}\) (SIK2) (13), and Ser\(^{171}\) (SIK3) (10, 19), which facilitates TORC-mediated activation of CREB. These findings indicate the importance of dephosphorylation of TORC in CREB-mediated gluconeogenesis processes.

Calcineurin (Cn) has been identified as the TORC phosphatase activated in response to Ca\(^{2+}\) signaling in pancreatic \(\beta\)-cells (35), and Ser\(^{275}\) of TORC2 has been found to be the sensitive site of this phosphatase (16). However, Ser\(^{171}\) phosphatase has not been identified, and the contribution of Cn to the activation of TORC in other cell types also remains unclear.

Protein phosphate 2A (PP2A) and protein phosphate 1 (PP1) are widely distributed phosphatases and play significant roles in the regulation of CREB-mediated gene expression (42). In addition, PP2A has been found to be an accessory protein of SIK2 in human kidney fibroblast human embryonic kidney (HEK)-293

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cells (1) and to be essential for SIK1-mediated activation of sodium/potassium ATPase in opossum kidney cells (39).

To clarify the complicated mechanisms of dephosphorylation-dependent activation of TORC2, we first used a conventional cultured cell line (COS-7) and noticed some discrepancies between the intracellular localization of TORC2 and its transcription activities. Experiments using the PP2A/PPI inhibitor okadaic acid (OA), the Cn inhibitor cyclosporin A (CsA), and TORC2 mutants provided evidence of the existence of a third TORC2 phosphorylation site, Ser\(^{307}\). Dephosphorylation at Ser\(^{171}\), Ser\(^{275}\), and Ser\(^{307}\) was differentially modified by different conditions. OA inhibited dephosphorylation of TORC2 at Ser\(^{171}\), which disrupted the coactivator activity of TORC2 in the nucleus. On the other hand, CsA blocked phosphorylation at Ser\(^{307}\), retaining TORC2 in the cytoplasm. Dephosphorylation levels at Ser\(^{171}\) and Ser\(^{307}\) varied in the liver depending on fasting-fed conditions and insulin resistance, and treatments with both OA and CsA inhibited gluconeogenesis in insulin-resistant mice.

MATERIALS AND METHODS

Chemicals and reagents. Forskolin, ATP, A23187, staurosporine, culture medium (DMEM), an antibody against a catalytic subunit of PP2A, and an anti-Flag M2 IgG were purchased from Sigma Aldrich (St. Louis, MO); OA, CsA, and glutathione columns were from Wako Pure Chemicals (Osaka, Japan), Calbiochem (San Diego, CA), and GE Healthcare UK (Buckinghamshire, UK), respectively. Anti-CREB antibodies were obtained from Cell Signaling Technology (Danvers, MA), anti-14-3-3 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-TORC2 antibodies were used as described elsewhere (19). Anti-rabbit Alexa fluor 488 and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Carlsbad, CA).

Anti-pTORC2 (p-Ser\(^{275}\)) and anti-pTORC2 (p-Ser\(^{307}\)) were generated in rabbits against phosphoprotein that had been conjugated with keyhole limpet hemocyanin (Ser\(^{275}\): CPANMTGGpSLPDLTNL; Ser\(^{307}\): CPSLSGGGpSLPDLTNL). To elminate nonspecific reactions, nonphosphorylated peptides corresponding to the sequences of Ser\(^{275}\) and Ser\(^{307}\) were prepared in vivo as fusion proteins of keyhole limpet hemocyanin (Ser275: CPANMTGGpSLPDLTNL; Ser307: CPSLSGGGpSLPDLTNL). To eliminate nonspecific interactions, anti-TORC2 antibodies were used as substrates for SIK1. To elute the Cn inhibitor cyclosporin A (CsA), phospho-Ser\(^{275}\) and phospho-Ser\(^{307}\) were prepared in vivo as fusion proteins of keyhole limpet hemocyanin (Ser275: CPANMTGGpSLPDLTNL; Ser307: CPSLSGGGpSLPDLTNL).

Expression vectors for human Cn (clone ID 19600441195878), PP2A regulatory subunit A (PPP2R2A; clone ID 59365353), PP2A regulatory subunit B (PPP2RB1B; clone ID 6067682), PP2A catalytic subunit (PPP2CA; clone ID 6016280), and PPI catalytic subunit (PPPI1C; clone ID CS0D3777YG01) were purchased from Invitrogen and introduced into the adenovirus vector pAd/CMV/V5-DEST using the Gateway system (Invitrogen). To prepare constitutively active Cn, the cDNA for the catalytic domain was amplified by PCR with the primers 5′-AAATTGATAGTCCGAGGCCCAGCCAAGGA and 5′-AATTGGCCGGCCCTACTTGGCTCCTATCAC, digested with BamHI and NotI, and ligated into the pENTR1A vector.

To examine mRNA levels in the liver, total RNA was purified with the EZ1 RNA Tissue Kit (Qiagen, Valencia, CA) and used as the template for the reverse transcription (RT) reaction with the Transcriptor Kit (Roche Diagnostic, Basel, Switzerland). The resultant cDNA was used for real-time polymerase chain reaction (PCR) analyses with Platinum SYBR Green qPCR SuperMix (Invitrogen) supplemented with 10% dimethyl sulfoxide. Real-time PCR analyses were performed using the MyiQ real-time PCR system (Bio-Rad Laboratories, Hercules, CA). Primers used in this study were made by Integrated DNA Technologies, Inc. (Coralville, IA) and were designed to amplify 150 bp long fragments.

Immunoprecipitation, in vitro kinase assay, and reporter analyses. Immunoprecipitation and in vitro kinase assays were performed as described previously (25). To separate the nuclei from the cytoplasmic fractions, the cells were lysed in IP lysis buffer supplemented with 0.1% Triton X-100, and the nuclei were precipitated by centrifugation at 3,000 rpm for 5 min. After separation, the nuclear proteins were eluted by addition of NaCl (final concentration 500 mM), and contaminated DNA was cut by sonication. The SIK1 enzyme was prepared in COS-7 as a GST fusion protein, and GST fusion peptides prepared as antibody absorbents were used as substrates for SIK1. To prepare a substrate for full-length TORC2, pGEX-mTORC2 was transformed into E. coli BL21. GST-TORC2 peptide was then induced using 0.2 mM isoprrol 1- (β-D-thiogalactopyranoside at 25°C for 3 h and purified on a glutathione column. Degraded peptides were removed by filtration using a Microcon-100 (Millipore, Billerica, MA).

To measure reporter activity, we used the Dual-Luciferase Reporter Assay System (Promega). The cells were lysed with 100 µl of passive lysis buffer, and 10 µl was used for the assay. The activities of firefly luciferase were normalized by those of Renilla luciferase, and the specific activities of CRE and TORC2 were expressed as a multiple of the control.

Cytochemical and immunohistochemical analyses. For fluorescent microscope observation, COS-7 cells were cultured on 18-mm poly-l-lysine-coated coverslips (Matsumani, Tokyo, Japan) using a 12-well dish. Cells that had been transformed with plasmids for green fluorescent protein (GFP)-TORC2 (0.5 µg) and for effectors (0.25 µg), e.g., PP1 and SIK1, were fixed with 1 ml of 4% paraformaldehyde dissolved in PBS for 15 min, stained with DAPI (1 ng/ml in 0.01% Triton X-100-PBS) for 5 min, and then washed with PBS four times. Cells on the coverslip were embedded on a slide glass using 50% glycerol. Several pieces of mouse liver (~50 mg) were fixed with 1 ml of 4% paraformaldehyde dissolved in PBS for 2 h and then immersed in 10% sucrose-PBS overnight. Five-micrometer sections were prepared as frozen sections using Tissue-Teck (Sakura, Tokyo, Japan) and mounted on glass slides coated with poly-l-lysine. After the specimens were blocked with a blocking solution (1:1 mixture of TBST:BlockOne supplemented with 1% BSA; Nakarai, Kyoto, Japan), they were incubated with primary antibodies (anti-TORC2 IgG, anti-PPI IgG) and then with secondary antibodies conjugated with appropriate fluorophores.
Fig. 1. Okadaic acid (OA) and cyclosporin A (CsA) differentially inhibit transducer of regulated cAMP response element-binding protein (CREB) activity (TORC2).

A: COS-7 cells were transformed using pEBG vectors for glutathione-S-transferase (GST) fusion salt-inducible kinases (SIKs; 2 μg) together with pCMV-Sport6 vectors for the protein phosphate 2A (PP2A) complex [0.5 μg each of three subunits: PPP2Ca (PP2A catalytic subunit), PPP2R1B (PP2A regulatory subunit A (RegA)), and PPP2R2A (PP2A regulatory subunit B)] and the pcDNA3.2 vector for calcineurin (Cn; full-length Cn, pCMV-Sport6, 0.5 μg). GST-SIKs were purified on a glutathione column (CP) and subjected to Western blot analyses. An antibody against PPP2R1B (RegA) was used to detect PP2A association. *Unknown band recognized by an anti-GST antibody.

B: COS-7 cells, transformed using the expression vector for GST fusion TORC2 (pEBG-TORC2, 5 μg) with or without the SIK1 expression vector (pSVL-HA-SIK1, 2 μg), were treated with 100, 300, or 1,000 nM OA or 10 μM CsA for 3 h. GST-TORC2 or HS-SIK1 was purified on a CP or with an HA-sepharose resin [immunoprecipitate (IP)] and subjected to Western blot analyses. CREB was detected with the aid of cell lysates. Four to twenty percent gradient gels were used to focus the phosphorylation signals of TORC2. A representative set of images from duplicate experiments is shown.

C: cAMP response element (CRE) activity was monitored using the CRE reporter pTAL-CRE. COS-7 cells were transformed using the vector for the CRE reporter (pTAL or pTAL-CRE, 150 ng) together with the internal Renilla reporter (pTK-Int, 30 ng). After 36 h, OA (100, 300, or 1,000 nM) or CsA (10 μM) was added to the culture medium in the presence or absence of forskolin (Fsk; 20 μM). The specific CRE activity is expressed as a multiple of the activity of the empty reporter pTAL.

D: CREB activity was monitored using a Gal4-fusion one-hybrid system. COS-7 cells were transformed using the vector for Gal4-fusion bZIP-less CREB (pM-CREB S, 100 ng) together with the firefly luciferase promoter possessing 5 repeats of the Gal4-binding site (pTAL-GAL4, 150 ng) and pTK-Int (30 ng). After 36 h, Fsk (20 μM) was added to the culture medium to activate CREB. E: a similar reporter assay was performed using the vector for Gal4-fusion NH2-terminal-less TORC2 (pM-TORC2 Δ45). Means and SD for triplicate experiments are shown in C–E. F: the intracellular distribution of TORC2 was examined using green fluorescent protein (GFP) fusion techniques. After the cells were fixed with paraformaldehyde, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells from duplicate transformations are shown expressing representative patterns for each treatment. Cont, control.
1/1,000 dilution; anti-Flag M2 IgG, 1/1,000 dilution) at 4°C for 12–16 h. After washing with TBST, the specimens were incubated with secondary antibodies (anti-rabbit for TORC2 or anti-mouse for Flag-tag), conjugated with Alexa fluor 488 (Invitrogen) at 1/300 dilution for 3 h, stained with DAPI, and washed with PBS. Blue signals for DAPI (nuclei) were changed to red with the image software ACT-1 and merged with green signals for TORC2. Quantification of bands on Western membranes was performed by the software Quantity One (Bio-Rad).

Mice. Experimental protocols for mice were approved by committees at the National Institute of Biomedical Innovation. C57BL/6J and Ay mice were obtained from SLC Japan (Shizuoka, Japan), and KK-Ay and db/db mice were from CLEA Japan (Tokyo, Japan). Mice were maintained under standard conditions of light (8 AM-8 PM) and temperature (23°C/40% humidity). All experiments were carried out in accordance with the guidelines for animal care of the National Institute for Biomedical Innovation. Blood glucose was measured with a kit from Wako Pure Chemicals. A high-fat diet (D12492, 60 kcal% fat) was acquired from Research Diets (New Brunswick, NJ). OA and CsA were first dissolved in DMSO and then diluted 100× with saline and administered as a single tail vein injection at doses of 50 μg/kg and 5 mg/kg, respectively. Isoflurane was used for anesthesia. Adenoviruses were purified using the BD Adeno-X Virus Purification kit, diluted to 10^{11} particles/ml, and administered as a single tail vein injection (10 ml/kg).

Statistical analysis. Data are presented as means ± SD. Statistical differences between means were evaluated using a two-tailed unpaired Student t-test. A P value <0.05 was considered statistically significant.

RESULTS

OA stabilizes phosphorylation of TORC2 at Ser^{171} but results in relatively small changes in the intracellular distribution of TORC2 in COS-7 cells. Because PP2A and Cn were thought to modulate signaling cascades involved in SIK/TORC/CREB systems (1, 35, 39), we wanted to confirm the

Fig. 2. The effects of overexpression of phosphatases on CREB/TORC2 activities and the intracellular distribution of GFP-TORC2 in COS-7 cells. A: COS-7 cells were transformed using the CRE reporter set (pTAL or pTAL-CRE, 150 ng; pTK-Int, 30 ng) together with expression vectors (100 ng) for SIK1, PP1ca (PP1 catalytic subunit), PP2Aca (PP2A catalytic subunit), and constitutively active Cn (kinase domain, 1–399aa). After 16 h, Fsk (20 μM) was added to the culture medium to activate CREB, and the cells were incubated for 6 h (n = 3, SD shown). Similar reporter assays were performed using the vectors for Gal4-fusion βZIP-less CREB (B) and Gal4-fusion NH1-terminal-less TORC2 (C) without Fsk activation (n = 2, bars indicate differences). D: GFP-TORC2 was overexpressed with SIK1 and phosphatases in COS-7 cells.

Fig. 3. Nuclear localization of TORC2 is not sufficient for its coactivator activity. A: COS-7 cells cotransformed with pGFPC-TORC2 and pTarget-SIK1 were fixed with paraformaldehyde. B: diagrams of TORC2 mutants used for assays of intracellular distribution and reporter analyses. CBD, CREB-binding domain; NES, nuclear export signal; CAD, constitutively active domain. C: the coactivator activity of TORC2 was examined as in Fig. 1C with and without the SIK1 expression vector pTarget-SIK1 (100 ng; n = 3).

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Differential Dephosphorylation of TORC2
existence of direct interactions between SIK family enzymes and these phosphatases. Full-length SIK1–3 or its kinase domain was tagged with GST, coexpressed with a mix of the PP2A complex (catalytic and regulatory subunits) and Cn in COS-7 cells, purified on glutathione columns, and subjected to Western blot analyses (Fig. 1A). Because the sensitivity of the antibody against the PP2A regulatory subunit A (RegA) was higher than the antibodies against the other subunits, we used anti-PP2A (RegA) to detect PP2A subunits. PP2A (RegA) coprecipitated with all of the SIKs, whereas Cn was not enriched among the precipitants. PP2A (RegA) may specifically bind to the COOH-terminal regulatory domain of the SIK(s) because it was not coprecipitated with the kinase domain. Therefore, we surmised that, like Cn, PP2A might be able to modulate TORC activities, and we proceeded to examine the effects of phosphatase inhibitors on TORC2 phosphorylation levels.

OA is the inhibitor most widely used in studies of PP1 and PP2A, and CsA is often used in studies of Cn (40). Therefore, we chose OA and CsA to examine the involvement of PP2A and Cn in the regulation of TORC2. Treatment of COS-7 cells with OA (100–1,000 nM) resulted in enhanced phosphorylation of CREB at Ser133, whereas stabilization of TORC2 phosphorylation at Ser171 required a high dose of OA (1,000 nM) (Fig. 1B). Interestingly, phosphorylation of SIK1 at Ser577, which inhibits the TORC phosphorylation activity of SIK1 (41), was elevated in COS-7 cells treated with a low dose of OA, suggesting that OA may somehow influence the SIK/TORC/CREB system through a relatively complex mechanism. The Cn inhibitor CsA produced a smaller change than did OA in the phosphorylation levels of TORC and CREB.

To elucidate the complex effects of OA, we examined the transcription activities of CREB and TORC2. Because the CREB-TORC2 complex is activated by PKA, we used forskolin (Fsk) to induce the cAMP-PKA cascade. When a CRE reporter was used to evaluate the CREB-TORC complex, Fsk-induced reporter activity was initially enhanced by 100 nM of OA, after which it was weakened by the compound at 1,000 nM (Fig. 1C). CsA, on the other hand, consistently suppressed CRE reporter activity (also see Supplemental Fig. S1; Supplemental Material for this article can be found on the AJP-Endocrinology and Metabolism web site). Similar results were obtained for the human hepatocarcinoma cell HepG2 (Supplemental Fig. S2). The transcription activity of bZIP-less CREB lacking the TORC-binding site was upregulated by OA at both doses (Fig. 1D). In contrast to CREB activity, high-dose OA suppressed the activity of NH2-terminal truncated TORC2, which lacked the CREB-binding site (Fig. 1E). These results suggest that CRE activity may largely reflect the transcription activity of TORC. CsA and high-dose OA suppressed CRE reporter activity probably by inhibiting the same target, i.e., TORC.

Because CsA blocks nuclear entry of TORC2, we examined the intracellular distribution of TORC2 using the GFP fusion technique (Fig. 1F) and found that CsA enhanced cytoplasmic localization of TORC2, whereas OA at any dose had a less pronounced effect. A small inconsistency between the intracellular distribution of GFP-TORC2 and the transcription activity of Gal4-TORC2 in COS-7 cells treated with OA may have been due to the different sensitivity of the assays or to complex regulatory mechanisms affecting TORC2.

PP2A and PP1 have opposite effects on the activity of TORC2 in COS-7 cells. The activity of PP2A in cultured cells has been reported to be blocked by a low dose of OA (40); however, PP1 is also inhibited by OA at a high dose. To examine the possible involvement of both PP2A and PP1 in the
biphasic response of CRE activity to different concentrations of OA (Fig. 1), we used overexpressions of the catalytic subunit of PP2A (PP2Aca), PP1 (PP1ca), and COOH-terminal truncated Cn (a constitutively active form) for reporter analyses (Fig. 2A). In addition, SIK1 was used as a TORC2 kinase, which resulted in a significant decrease in CRE reporter activity under both basal and Fsk-induced conditions. When PP1ca was overexpressed, CRE reporter activity was upregulated, whereas overexpression of PP2Aca resulted in a decrease in activity. Unexpectedly, overexpression of Cn failed to modulate CRE activity. In contrast to the assays using OA and CsA (Fig. 1, C–E), both basal and Fsk-induced CRE activity were modulated in parallel by SIK1 and the phosphatases, suggesting that overexpression of these factors may modulate CREB and TORC2 activities more specifically than inhibitors.

In a manner similar to OA and CsA (Fig. 1, C, D, and F), we proceeded to examine the effects of SIK1 and phosphatases on the transcription activities of Gal4-fusion CREB and TORC2. Because Fsk diminished the effect of SIK1 on Gal4-TORC2 due to PKA-dependent phosphorylation at Ser577 (19), we evaluated the basal activities of Gal4-fusion CREB and TORC2 and compared them with the CRE activity. When Gal4-bZIP-less CREB was used in the reporter analysis (Fig. 2B), both PP1ca and PP2Aca suppressed CREB activity. However, when Gal4-TORC2 was used as the reporter, PP1ca upregulated TORC2 activity, whereas PP2Aca suppressed it (Fig. 2C). Overexpression of constitutively active Cn failed to produce any substantial differences. SIK1 repressed TORC2 but not CREB, suggesting that the response of the CRE reporter may reflect the activity of endogenous TORC.

Next, we examined the effects of overexpression of SIK1 and phosphatases on the intracellular distribution of TORC2 (Fig. 2D). SIK1 and PP2A enhanced cytoplasmic retention of GFP-TORC2, whereas Cn produced nuclear-specific accumulation of GFP-TORC2. Overexpression of PP1 induced slight nuclear accumulation of GFP-TORC2. These results and those shown in Fig. 1 suggest that low-dose OA may initially inhibit PP2A, and this inhibition may then inactivate TORC kinases such as SIK1 by stabilizing phosphorylation at Ser577, which eventually results in upregulation of TORC activity. On the other hand, high-dose OA concomitantly inhibits PP1, which results in inactivation of TORCs by stabilizing phosphorylation, such as at Ser171 of TORC2. However, we cannot explain the discrepancy between the intracellular distribution of TORC2 and its transcription activity in cells treated with OA (Fig. 1, E and F) or overexpression of Cn (Fig. 2, C and D).

The coactivation activity of TORC2 is not related to its nuclear localization in COS-7 cells. PP1 may dephosphorylate TORC2 at Ser171 and activate CREB-dependent transcription; however, nuclear entry of TORC2 may not be coupled with phosphorylation at Ser171. Ser275 is located at the NH2 terminus of the nuclear export signal (NES) of TORC2, and its phos-
phorylation and that of Ser\textsuperscript{171} were found to cooperatively enhance cytoplasmic localization of TORC2. Therefore, to investigate intracellular distribution, we prepared representative TORC2 mutants (Fig. 3, A and B) and compared their distributions to their coactivator activities (Fig. 3C).

A double mutation at Ser\textsuperscript{171} and Ser\textsuperscript{275} increased the nuclear distribution of TORC2 but resulted in incomplete inhibition of SIK1-induced cytoplasmic localization of TORC2 (Fig. 3A). On the other hand, the coactivator activity of the S171A/S275A mutant was not strongly suppressed by SIK1 (Fig. 3C). Truncation of the NES with Ser\textsuperscript{275} (A275–280) retained TORC2 in the nucleus irrespective of SIK1, whereas its coactivator activity was strongly repressed by SIK1.

Ser\textsuperscript{307} is essential for nuclear export of TORC2 in COS-7 cells. Although nuclear export of TORC2 may be important for SIK1-mediated suppression of the coactivator activity of TORC2, double substitution of S171A/S275A failed to completely inhibit SIK1-induced nuclear export. Therefore, we decided to examine whether unidentified SIK1-dependent phosphorylation sites were present, presumably near the NES. SIK1 was able to phosphorylate Ser but not Thr, and three Ser phosphorylation sites were present, presumably near the NES. SIK1-mediated nuclear export of TORC2 (Fig. 3B). Without SIK1 overexpression, nuclear localization of TORC2 was enhanced by a single substitution at S171A or S275A. However, these substitutions were insufficient to block SIK1-mediated nuclear export of TORC2 (Fig. 4B). S303A substitution had no effect on the intracellular distribution of TORC2, whereas S307G substitution completely localized TORC2 in the nucleus irrespective of SIK1, whereas its coactivator activity was strongly repressed by SIK1.

Because various signaling cascades may induce dephosphorylation of TORC, we decided to monitor cascade-dependent dephosphorylation of TORC2 in SIK1-overexpressing COS-7 cells (Fig. 5A). Ca\textsuperscript{2+} and cAMP signals were induced using A23187 and Fsk, respectively; staurosporine (STS), which potently inhibits TORC kinases, including SIKs, was used to prepare dephospho-TORC2. Fsk induced dephosphorylation at Ser\textsuperscript{171} and Ser\textsuperscript{275} more efficiently than did A23187, whereas dephosphorylation at Ser\textsuperscript{307} was preferentially preformed by A23187. STS reduced phosphorylation levels at all sites. The level of 14-3-3 binding apparently correlated with the level of the phospho-Ser/Thr accessory protein 14-3-3.

Fig. 6. Modulation of the intracellular localization of TORC2 in the liver by chemicals and phosphatases. After starvation (24-h fasting), OA (50 µg/kg) or CsA (5 mg/kg) was administered with a single tail vein injection into 12-wk-old C57BL/6J mice. After 1 h, the liver was resected for gluconeogenic gene expression analysis (A). Western blot analysis (B), and immunohistochemistry (C). *P < 0.05 compared with the control group (DMSO; n = 3 for each group). B, right: quantification of the bands on Western blot membranes is shown (bars indicate SD). Nuclei were stained with DAPI, and the blue signals for DAPI were changed to red using the image software ACT-1 and merged with the green signals for TORC2. C, insets: higher-magnification images of immunohistochemistry are shown.
The effects of OA and CsA on TORC2 phosphorylation of each of the residues were also examined after separation of nucleic and cytoplasmic fractions (Fig. 5E). OA stabilized phosphorylation at Ser\textsuperscript{171}, but not at Ser\textsuperscript{275} or Ser\textsuperscript{307}, in both the nucleus and the cytoplasm, whereas CsA enhanced phosphorylation at Ser\textsuperscript{307} of cytoplasmic TORC2. Overexpression of SIK1 and addition of STS were used as positive and negative controls. These findings suggest that suppression of the transcription activity of TORC2 and its nuclear export may be initiated by phosphorylation at different sites by different kinases under various conditions. However, under overexpression conditions for SIK1 and MARKs, they may phosphorylate any of the sites equally. In contrast to phosphorylation, dephosphorylation of the various sites may be differentially performed by the specified phosphatase at each site.

**Phospho/dephosphoregulation of TORC2 at Ser\textsuperscript{171} and Ser\textsuperscript{307} in the mouse liver.** Dephosphorylation at Ser\textsuperscript{171} of TORC2 is important for gluconeogenesis in the liver, and SIK1 has been identified as a fasting regulator of TORC2. Therefore, we decided to monitor other phosphorylation sites, Ser\textsuperscript{275} and Ser\textsuperscript{307}, in the mouse liver after treatments with OA and CsA. Because insulin has been reported to regulate the activity of TORC2, C57BL/6 mice were fasted for 24 h to reduce insulin levels, after which they were injected with OA or CsA via the tail vein, and the liver was resected 1 h posttreatment.

Gene expression involved in the gluconeogenic program, especially PGC-1\(\alpha\), was suppressed by OA and CsA (Fig. 6A). TORC2 phosphorylation at Ser\textsuperscript{171} was stabilized by OA, and phospho-Ser\textsuperscript{307} was enhanced by CsA (Fig. 6B). We were not able to detect any changes in the phosphorylation level at

Fig. 7. The effects of overexpression of SIK1 and phosphatases on the intracellular localization of TORC2 in the liver. Adenoviruses for SIK1 and phosphatases were administered to 8-wk-old C57BL/6j mice (n = 3). After 16 h, 1 group (LacZ, SIK1, and the catalytic subunit of PP2A; A and C) was subjected to fasting for 24 h, and the other group (LacZ, the catalytic subunit of PP1, and the kinase domain of Cn; B and D) was fed ad libitum. Western blot analyses and immunohistochemistry were performed as in Fig. 6.
compounds, we may be able to regulate gluconeogenic proteolytic activity of TORC2 (Fig. 9). These results suggest that, using small Eplasm, but OA had less effect on the intracellular distribution of SIK1 and PP2A resulted in retention of TORC2 in the cytoplasm, accompanied by enhanced phosphorylation at Ser\(^{171}\) and Ser\(^{307}\) (Fig. 7C). On the other hand, PP1 and Cn induced nuclear localization of TORC2, accompanied by a reduction in the level of phospho-Ser\(^{307}\) (Fig. 7D). Ser\(^{171}\) levels were reduced in liver tissues expressing PP1 but not Cn.

To evaluate the direct roles of each phosphorylation site in the regulation of coactivator activity and intracellular localization of TORC2, Flag-tagged TORC2 and associated mutants were overexpressed in the mouse liver, and gluconeogenic gene expression and localization of TORC2 were examined. As shown in Fig. 8A, all of the TORC2s enhanced gluconeogenic gene expression in the mouse liver under fed conditions. Ser\(^ {171}\)-disrupted TORC2 induced mRNA(s) more efficiently than wild-type or other mutant TORC2s. When we monitored the phosphorylation levels at each site (Fig. 8B), we noticed that disruption of one phosphorylation site did not alter the phosphorylation levels of other sites, suggesting that the levels at each site might be regulated independently. Immunohistochemical analyses revealed that only Ser\(^ {307}\)-disrupted TORC2 was localized in the nucleus (Fig. 8C). These results suggest that Ser\(^ {307}\) is important for intracellular localization of TORC2 in COS-7 cells and in the mouse liver.

**Hepatic TORC2 in diabetic animals.** Because activation of TORC2 is reportedly enhanced in the livers of diabetic animals (22), we examined the intracellular distribution of TORC2 in the livers of obese and obese/diabetic mice. KK-A\(^ {v}\) mice have strong insulin resistance due to polymeric disorders of insulin sensitivity (KK) associated with overeating (A\(^ {v}\)) (33). A\(^ {v}\)-only mice are obese but not insulin resistant, whereas db/db mice are insulin resistant due to the lack of the leptin receptor (3). Under fed conditions, the intracellular localization of TORC2 in two obesity models (high-fat diet-induced obesity and A\(^ {v}\)) was indistinguishable from that in normal C57BL/6 mice (Fig. 9A). In contrast, TORC2 was localized mainly in the nuclei of the livers of KK-A\(^ {v}\) and db/db mice. Figure 9B shows blood glucose levels under fed conditions.

Finally, we investigated whether OA and CsA modified the activity of TORC2 in the livers of diabetic animals. Although the blood glucose levels of KK-A\(^ {v}\) mice were less affected by administration of OA or CsA [Supplemental Fig. S3; levels of insulin and alanine aminotransferase (glutamic-pyruvic transaminase) are also shown in Supplemental Figs. S4 and S5], gluconeogenic gene expression in the liver was suppressed by these chemicals (Fig. 9C). TORC2 phosphorylation at Ser\(^ {171}\) was stabilized by OA, and phospho-Ser\(^ {307}\) was enhanced by CsA (Fig. 9D). CsA retained TORC2 in the cytoplasm, but OA had less effect on the intracellular distribution of TORC2 (Fig. 9E). These results suggest that, using small compounds, we may be able to regulate gluconeogenic programs via differential modification of the phosphorylation status of TORC2. It should be noted that overexpression of neither SIK1 nor PP2A induced cytoplasmic localization of TORC2 in the livers of KK-A\(^ {v}\) mice. This result agrees with our previous finding that levels of SIK1 mRNA were already high in the livers of db/db mice, suggesting the possibility of SIK1 resistance in the livers of diabetic animals (13).

**DISCUSSION**

Nuclear cytoplasmic shuttling of TORC2 is a critical mechanism of its coactivator activity (2, 20, 35). Ser\(^ {171}\) was first identified as a phosphorylation site regulating both the intracellular distribution and coactivator activity of TORC2 (35). However, we and other groups have found several contradictory relationships between the intracellular distribution and coactivator activity of TORC2 and have succeeded in resolving these contradictions by identifying differential dephosphorylation at different Ser residues (16).

The second phosphorylation site is Ser\(^ {275}\) (23). Glucose metabolism in pancreatic β-cells produces ATP, which closes the ATP-sensitive K\(^ {+}\) channel (36), inducing membrane depolarization followed by opening of the Ca\(^ {2+}\) channel. The
resultant increase in cellular Ca\(^{2+}\) activates Cn, which initiates glucose-dependent dephosphorylation of TORC2 at Ser\(^{171}\) and Ser\(^{307}\), respectively, in the liver under fasting or insulin-resistant conditions. To prepare diet-induced obese (DIO) animals, 8-wk-old C57BL/6J mice were fed with high-fat (HF; 60 kcal% fat) diets for 2 mo. In addition to DIO, we used obese model (A\(^{y}\)) mice (12 wk old) and obese model mice with insulin resistance (KK-A\(^{y}\) and db/db). A: immunohistochemical analyses of TORC2 in the liver. B: blood glucose levels of the mice under ad libitum feeding conditions (n = 3; bars indicate SD). Twelve-week-old KK-A\(^{y}\) mice were given OA (50 \(\mu\)g/kg) or CsA (5 mg/kg) with a single tail vein injection. After 1 h, the liver was resected for gluconeogenic gene expression analyses (C), Western blot analyses (D), and immunohistochemistry (E). *P < 0.05 compared with the control group (DMSO; n = 3; bars indicate SD).

However, double substitution at Ser\(^{171}\) and at Ser\(^{375}\) failed to inhibit SIK1-mediated nuclear export of TORC2. To understand the molecular mechanism by which SIK1 induces the nuclear export of TORC2, we searched for other important phosphorylation sites and found Ser\(^{307}\). Disruption of Ser\(^{307}\) completely inhibited SIK1-induced nuclear export of TORC2 in COS-7 cells. Interestingly, the coactivator activity of the S307G mutant was still inactivated by SIK1. In addition to the S307G-TORC2 mutant, we reported previously that TORC3, whose nuclear export signal was impaired (Fig. 4A) (35), was localized predominantly in the nucleus of COS-7 cells, and this
localization was less influenced by SIK1 overexpression. However, when TORC3 was expressed as a Gal4 fusion protein, SIK1 overexpression resulted in a loss of transcription activity of Gal4-TORC3 (19), suggesting that nuclear localization of TORC is important for exerting its coactivator activity; however, other processes may also be required. In contrast to the activation processes of TORC2, either blocking nuclear import of TORC or suppressing its coactivator activity appears to be sufficient to inhibit CREB-mediated gene expression.

TORC1 is localized predominantly in the cytoplasm even in HeLa cells that lack TORC kinase activity due to deficiency in the upstream kinase LKB1 (19, 26). In these HeLa cells, the MAP kinase MEKK1 was found to contribute to nuclear import of TORC1 by phosphorylating (not dephosphorylating) the COOH-terminal constitutively active domain (38). MEKK1-dependent activation of TORC1 is insensitive to CsA. cAMP-induced nuclear import of TORC1 is also not blocked by CsA (2), suggesting that intracellular distribution of each of the isoforms of TORC may be regulated by different mechanisms. For example, disruption at Ser293 of TORC1, corresponding to Ser307 of TORC2, does not affect the intracellular distribution of TORC1 in COS-7 cells (data not shown).

Because the Cn inhibitor CsA stabilizes phosphorylation at Ser307, Cn may play an important role in the nuclear import of TORC2, probably through Ser307 dephosphorylation. However, CsA was able to inhibit CRE reporter activity induced not only by wild-type TORC2 but also by the nuclear mutant TORC2 (S307G) and TORC3 (data not shown). The presence of multiple CsA-sensitive sites in TORC2 in addition to Ser71 has been demonstrated by phosphopeptide mapping (35), suggesting that Cn may regulate TORC2 action via combined dephosphorylation.

The 14-3-3 protein family recognizes motifs of R/KSXpS/ptXP or RXXXpSXP (pS/pT represents phospho-Ser/Thr) and modulates various functions of target molecules, including nucleocytoplasmic shuttling (27). Ser307 does not fit into the 14-3-3 motif, whereas Ser275 possesses a Pro residue at the +2 position. Although treatment with a calcium ionophore (A23187) or CsA changed the level of association between 14-3-3 and TORC2, we could not obtain direct evidence that Ser307 was the 14-3-3 binding site.

The PP2A complex, consisting of a catalytic subunit and a regulatory subunit A/B, is reportedly associated with SIK2 in HEK-293 cells (1). Oddly, the gene ppp2r1b, which encodes the PP2A regulatory subunit A (PR65) β-isoform, is located on the antisense strand 3’ downstream of the sik2 gene, and these two genes evolutionally share their 3’-untranslated regions (18). The results of the copurification study suggested that the PP2A complex usually binds to SIK family kinases and probably plays an important role in the actions of SIKs. One of these actions may be dephosphorylation of SIK1 at Ser577, because a low dose of OA stabilizes phosphorylation, whereas overexpression of the PP2A catalytic subunit suppresses TORC2-mediated reporter activity.

On the other hand, high-dose OA, which probably inhibits not only PP2A but also PP1, suppressed CREB/TORC2-mediated gene expression, which was accompanied by stabilization of TORC2 phosphorylation at Ser171. Moreover, overexpression of the PP1 catalytic subunit activated the transcription activity of TORC2 in COS-7 cells and dephosphorylated TORC2 at Ser171 in the mouse liver. PP1 is believed to be a repressor of CREB because of its dephosphorylation of Ser133; however, it has recently been reported that PP1 is capable of inducing CREB-mediated gene expression independently of Ser133 phosphorylation in HEK-293 cells (9).

At 200 nM, OA is known to upregulate CREB-dependent transcription, including PEPCK gene expression, by enhancing phosphorylation of CREB at Ser133 in HepG2 cells (42). However, it has also been reported that 400 nM of OA is enough to suppress PEPCK gene expression in H4IE cells (30), suggesting that cell type, concentration, and lot number may determine OA actions on CREB-mediated transcription, probably due to low selectivity against a variety of phosphatases, including PP1 and PP2A.

Activation of CREB induces gluconeogenic gene expression in the liver, and SIK-TORC2 signaling partially explains the complicated regulation of gluconeogenic programs. During gluconeogenic gene expression, the gene for sik1 is also induced by cAMP-CREB during fasting, whereas the induced SIK1 phosphatolyses TORC2 and represses CREB-mediated gene expression, including that of its own gene, thus achieving negative feedback regulation of the gluconeogenic program (22). SIK1-mediated autophosphorylation of gluconeogenic programs is less affected by insulin signaling. On the other hand, SIK2 is activated by insulin signaling as a result of phosphorylation at Ser558 by Akt and inactivates TORC2, which attenuates gluconeogenic programs (6). However, studies of SIK2-knockout mice suggest that SIK2 contributes less to the PGC-1α-mediated regulation of gluconeogenic gene expression in the liver or that redundant actions of AMPK-related kinases may address the SIK2 deficiency (Takemori H, Nakai R, and Morita J, unpublished observations).

Previous findings of in vitro studies have suggested that MARK2 phosphatolyses TORC2 preferentially at Ser275 and that SIKs and AMPK do so efficiently at Ser71. Thus, it has been proposed that TORC2 is differentially phosphorylated at each Ser residue by SIK1/AMPK and MARK2 (8). However, in COS-7 cells, SIK1, MARK2, and MARK4 were all able to

Fig. 10. Hypothetical model of phospho/dephosphorylation of TORC2. SIK1 inactivates TORC2 by phosphorylation. The TORC2 phosphorylation activity of SIK1 is suppressed by Ser577 phosphorylation via PKA, which is restored by dephosphorylation via the SIK1-associated phosphatase PP2A. Three phosphorylation sites have been identified in COS-7 cells and in the mouse liver: Ser133, Ser275, and Ser307. Phosphorylation at Ser133 suppresses the coactivator activity of TORC2, and PP1 dephosphorylates this site. In contrast, phosphorylation at Ser307 inhibits the nuclear localization of TORC2, and Cn dephosphorylates this site. Compounds that modulate these processes are shown. Arrows indicate phosphorylation. Bars indicate dephosphorylation or inhibition.
phosphorylate the three Ser residues (Ser\textsuperscript{171}, Ser\textsuperscript{275}, and Ser\textsuperscript{307}). Therefore, we surmise that all TORC kinases may phosphorylate important phosphorylation sites with almost equal efficiency and that extracellular stimuli may differentially regulate each TORC kinase in a cell type-specific manner. In contrast to phosphorylation processes, dephosphorylation of TORC2 may be regulated by different phosphatases at different phospho-Ser residues to implement the complicated regulation of the gluconeogenic program in the liver. On the basis of previous and present studies, we present a hypothetical model of phospho/dephosphorylation of TORC2 in Fig. 10.

We have to mention here that although COS-7 cells (monkey kidney fibroblasts) are often used for overexpression experiments, they do not possess gluconeogenic activity and related signaling cascades. In addition, TORC2 is distributed in both the nucleus and the cytoplasm of resting COS-7 cells, which are different from other cells. HEK-293 (TORC2 is distributed not only in the cytoplasm but also in the nucleus in our resting HEK-293 cells) (15), and primary hepatocytes (22), suggesting that COS-7 cells are not suitable to analyze TORC2’s function. Therefore, we tried to examine dephosphorylation cascades and elucidate the importance of Ser\textsuperscript{307} in HepG2 cells or in primary cultured mouse hepatocytes. However, characters of HepG2 cells [aggregate, a variety of sizes of nuclei, and abnormal shape of organelles (lipid droplets)] make it difficult for us to judge precisely. In addition, we failed to culture primary mouse hepatocytes with gluconeogenic potency under a condition free of insulin or dexamethasone. As a result, we could not conclude whether compounds altered the distribution of TORC2 directly or indirectly, such as via modification of hormonal regulation. For example, AICAR-activated AMPK and insulin-activated SIK2 are also found to induce nuclear fragmentation of TORC2 in hepatocytes (6, 15). To examine direct actions of compounds and phosphatases on TORC2 in hepatocytes, we have to develop methods to culture hepatocytes with defined factors in future experiments.

Moreover, we failed to obtain clear evidence of intracellular distribution of TORC2 in the mouse liver by cell fractionation assay, probably due to contamination of different sizes of fragmented organelles and unstable character of TORC2 protein and its phosphogroups. To redeem nonquantitative nature of immunohistochemical analyses, we have to develop methods for quick cell fraction in future studies. These efforts may elucidate more precise mechanisms underlying the regulation of TORC2 in gluconeogenic programs in the liver.

Various TORC2 functions related to glucose metabolism in pancreatic β-cells (35), muscles (43), brown adipose tissue (28), and white adipose tissue (31) have been reported. Recently, glucose metabolism in the hepatocytes of TORC2-knockout mice was analyzed (23). Although gluconeogenic gene expression was lower in the livers of TORC2-deficient mice than in the controls, glucose homeostasis in the whole body was less affected in the mutant mice. Similarly, OA and CsA were found to lower blood glucose only a little. Further identification of molecular probes such as OA and CsA also can be expected to be helpful in understanding the differential regulation of TORC activities in different cell types.

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DISCLOSURES

We have no conflicts of interest, financial or otherwise, to declare.

REFERENCES

Differential Dephosphorylation of TORC2


