Pin1 Associates with and Induces Translocation of CRTC2 to the Cytosol, Thereby Suppressing cAMP-responsive Element Transcriptional Activity*5

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Pin1 is a unique regulator, which catalyzes the conversion of a specific phospho-Ser/Thr-Pro-containing motif in target proteins. Herein, we identified CRTC2 as a Pin1-binding protein by overexpressing Pin1 with Myc and FLAG tags in mouse livers and subsequent purification of the complex containing Pin1. The association between Pin1 and CRTC2 was observed not only in overexpression experiments but also endogenously in the mouse liver. Interestingly, Ser136 in the nuclear localization signal of CRTC2 was shown to be involved in the association with Pin1. Pin1 overexpression in HepG2 cells attenuated forskolin-induced nuclear localization of CRTC2 and cAMP-responsive element (CRE) transcriptional activity, whereas gene knockdown of Pin1 by siRNA enhanced both. Pin1 also associated with CRTC1, leading to their cytosol localization, essentially similar to the action of CRTC2. Furthermore, it was shown that CRTC2 associated with Pin1 did not bind to CREB. Taken together, these observations indicate the association of Pin1 with CRTC2 to decrease the nuclear CBP-CRTC-CREB complex. Indeed, adenoviral gene transfer of Pin1 into diabetic mice improved hyperglycemia in conjunction with normalizing phosphoenolpyruvate carboxykinase mRNA expression levels, which is regulated by CRE transcriptional activity. In conclusion, Pin1 regulates CRE transcriptional activity, by associating with CRTC1 or CRTC2.

Pin1 was initially cloned as a NIMA kinase-interacting protein (1). Since its discovery, numerous proteins have been identified as Pin1 substrates, including p53, cyclin D1, and Tau (2–5). Pin1 interacts with a number of target proteins through recognition of phospho-Ser/Pro motifs, and the proline conformational change induced by Pin1 modifies the structures and functions, such as stabilization, phosphorylation, and translocation, of target proteins (4–7). Pin1 possesses the WW and PPlase3 domains in its N-terminal (amino acids 1–38) and C-terminal (amino acids 39–163) regions, respectively. To date, many reports have supported an important role for Pin1 in diabetes such as cancer and Alzheimer disease (4, 5). In this study, we demonstrated that Pin1 is also involved in metabolic disease via regulation of CRTC2 (CREB-regulated transcriptional co-activator 2; also known as TORC).

The cAMP-responsive element (CRE)-binding protein (CREB) stimulates transcriptional activity through recruitment of the histone acetylase CBP and through an association with CRTC, leading to formation of the CREB-CBP-CRTC complex on a CRE site (8–16). Thus, multiple molecular mechanisms affect the CREB-CBP-CRTC complex, resulting in the regulation of CRE transcriptional activity. They include the phosphorylations of CREB at Ser133, CBP at Ser436 and CRTC2 at Ser171 (16, 17). The phosphorylation of CRTC2 at Ser171 reportedly leads to an association with 14-3-3 protein and thereby to its nuclear exclusion and degradation (16).

The CRTC family consists of three members, CRTC1, CRTC2, and CRTC3 (16, 18). CRTC1 is highly expressed in the brain, whereas the other two are ubiquitously expressed (19). In the liver, insulin induces the phosphorylation of CRTC2 at Ser171, and this phosphorylation leads to the aforementioned

3 The abbreviations used are: PPlase, peptidyl-prolyl cis-trans-isomerase; CRE, cAMP-response element; CREB, CRE-binding protein; NLS, nuclear localization signal; MEF, mouse embryonic fibroblast; STZ, streptozotocin; PEPCK, phosphoenolpyruvate carboxykinase; CRTC, CREB-regulated transcriptional co-activator.
association with 14-3-3 protein and the nuclear exclusion and degradation of CRTC2 (16, 20). In contrast, glucagon induces dephosphorylation of CRTC2 and translocation from the cytosol to the nucleus, thereby forming the CRE-BBP-CRTC2 complex and inducing gluconeogenesis (21). Thus, CRTC2 plays important roles in hepatic glucose metabolism.

In this study, we identified CRTC2 as a Pin1-binding protein. Interestingly, the portion of CRTC2 responsible for the association with Pin1 was revealed to be in the nuclear localization signal (NLS) domain. Herein, we demonstrate that Pin1 regulates the functions and subcellular localizations of CRTC family proteins, thereby altering CRE transcriptional activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-Pin1 antibody was generated by immunizing rabbits with the peptide QMVKPFDASFAFRTGEMSG-PVFDDGSHIIITRTE (amino acids 129–163 of human Pin1). Anti-FLAG tag and Myc tag antibodies were purchased from Sigma-Aldrich. The antibodies against CRTC2, CREB, 14-3-3 protein, GFP, and DsRed were purchased from Cell Signaling Technology. Anti-rabbit HRP antibodies conjugated to horseradish peroxidase were obtained from Amersham Biosciences. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were purchased from Invitrogen. All other reagents were of analytical grade.

**Preparation of Adenoviruses Expressing MEF-tagged Pin1, CRTC1, and CRTC2**—The Myc-TEV-FLAG (MEF) tag cassette was generated by DNA synthesis and inserted into cloning sites in the mammalian expression vector pcDNA3 (Invitrogen; termed pcDNA3-MEF). To create the N-terminally MEF-tagged Pin1 construct, human Pin1 cDNA was inserted into pcDNA3-MEF. Then the coding portion of MEF-tagged Pin1 was isolated from pcDNA3-MEF-Pin1, and the recombinant adenoviruses containing the cDNA coding for MEF-tagged Pin1 were constructed as described previously (22). Recombinant adenoviruses expressing human Pin1 with the C-terminal HA tag or N-terminal MEF tag were also constructed and used for adenoviral gene transfer to HepG2 cells and mouse liver. Similarly, adenoviruses expressing GFP-tagged CTRC1, CRTC2, and GFP-tagged CRTC2 were prepared. Adenovirus encoding LacZ served as a control, and the adenoviral gene transfer was performed as reported previously (22).

**Purification of MEF-tagged Pin1 from Mouse Livers**—Recombinant adenovirus expressing MEF-tagged Pin1 was generated, purified, and concentrated using cesium chloride ultracentrifugation as reported previously (22). Adenovirus encoding LacZ served as a control. Male mice, 9 weeks of age, were obtained from the Nippon Bio-Supp. Center (Tokyo, Japan). They were infected, via the tail vein, with adenovirus at a dose of 2.5 × 10^9 particles/ml. The mice were injected with 100 μl of anti-Myc-conjugated Sepharose beads for the first immunoprecipitation. After incubation for 90 min at 4 °C, the beads were washed five times with 1.5 ml of TNTG buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (w/v) glycerol, 0.1% (w/v) Triton X-100), twice with buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% (w/v) Triton X-100), and finally once with TNT buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (w/v) Triton X-100). The washed beads were incubated with 15 units of TEV protease (Invitrogen) in 150 μl of TNT buffer to release bound materials from the beads. After incubation for 60 min at room temperature, the supernatant was pooled, and the beads were washed twice with 75 μl of buffer A. The resulting supernatants were combined and incubated with 25 μl of FLAG-Sepharose beads for the second immunoprecipitation. After incubation for 60 min at room temperature, the beads were washed three times with 500 μl of buffer A, and proteins bound to the FLAG beads were dissociated by incubation with 1 mM synthetic FLAG peptides in buffer A for 120 min at 4 °C. Approximately 3 μg of protein (0.01% of starting materials) were routinely recovered by this procedure. The samples were electrophoresed and subjected to SDS-PAGE and immunoblotting.

**Cell Culture**—SF9 cells were grown in TC100 (Invitrogen) medium containing 10% fetal calf serum at 27 °C. HepG2 hepatoma cells were grown in DMEM containing 10% fetal calf serum at 37 °C in 5% (v/v) CO₂ in air.

**Preparation of Baculoviruses Expressing Pin1 and CRTC2 Constructs**—The full-length coding regions of human Pin1, GFP, GFP-tagged Pin1, CRTC2, and DsRed-tagged full-length and various deletion mutant forms of CRTC2 and S136A CRTC2 were subcloned into pBacPAK9 transfer vector (Clontech), and the baculoviruses were prepared according to the manufacturer’s instructions. For protein production, SF9 cells were infected with these baculoviruses and grown for 48 h.

**Preparation of Glutathione S-Transferase (GST)-Pin1 Fusion Protein**—The cDNAs encoding full-length human Pin1, the WW domain of Pin1, and the PPlase domain of Pin1 were subcloned into a pGEX-5X-1 vector (Amersham Biosciences), which was used to transform *Escherichia coli* JM105 (Promega). Transformed cells were grown to an OD₆₀₀ of 0.6 in LB medium supplemented with 0.1 mg/ml ampicillin and stimulated for 3 h with 1.0 mM isopropyl-β-D-thiogalactopyranoside. GST fusion proteins were conjugated to glutathione-Sepharose 4B (Amersham Biosciences) and used for GST pull-down experiments.

**GST Pull-down**—HepG2 cells expressing MEF-CRTC and its mutants were homogenized with homogenizing buffer (20 mmol/liter Tris/HCl (pH 7.4), 1% Triton X-100, 0.25% sodium deoxycholate, 0.25 mol/liter NaCl) containing 0.2 mmol/liter phenylmethylsulfonyl fluoride and 5 μg/ml aprotinin and centrifuged at 15,000 rpm for 30 min at 4 °C, and the supernatants were then recentrifuged at 100,000 × g for 1 h. The supernatants (2 μg/ml protein concentration) were incubated with 1 ml of glutathione-Sepharose 4B for 1 h at 4 °C to remove nonspecifically bound proteins and then incubated with purified GST alone, GST-Pin1, and GST-Pin1 deletion mutant proteins for 1 h and finally washed six times with homogenizing buffer. Glutathione-Sepharose 4B beads
were boiled in Laemmli sample buffer, which was used for the SDS-PAGE and immunoblotting.

Preparation of Streptozotocin-treated Diabetic Mice and Gene Transfer of Pin1 into Mouse Livers—Streptozotocin (STZ)-treated diabetic male C57BL/6 mice (8–10 weeks of age) were prepared as reported previously (20). These mice were injected, via the tail vein, with adenovirus at a dose of 2.5 × 10^7 plaque-forming units/g body weight. Animals were fasted for 14 h and then were refed for 4 h before sacrifice. Blood glucose was measured with a portable blood glucose monitor, Glutest-Ace (Sanwa Kagaku Kenkyusho, Nagoya, Japan). All animal studies were conducted according to the Japanese guidelines for the care and use of experimental animals.

Immunoprecipitation and Immunoblotting—For the immunoprecipitation experiments, whole-cell extracts from HepG2 or SF9 cells or mouse liver lysates obtained after an overnight fast were prepared in lysis buffer, as described above. Cell or tissue extracts were incubated for 4 h at 4 °C with the indicated antibody and then for 1 h with 30 μl of protein G-Sepharose beads. The pellets were washed five times with 1 ml of lysis buffer and then resuspended in Laemmli sample buffer, boiled for 3 min, and analyzed on SDS-polyacrylamide gels.

Western blot analysis was carried out as described previously (22). In brief, 10 μg of protein were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes in a transfer buffer consisting of 20 mM Tris-HCl, 150 mM glycine, and 20% methanol. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 and incubated with specific antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody interactions were visualized by incubation with ECL chemiluminescence reagent (Amersham Biosciences).

Immunostaining—HepG2 cells were fixed with 4% paraformaldehyde for 10 min, rinsed with phosphate-buffered saline (PBS), and then exposed to 0.2% Triton X-100 in PBS for 5 min. Cells were subsequently incubated for 1 h at room temperature with anti-rabbit CRT2C (1:500), and fluorescein isothiocyanate-labeled secondary antibodies were added. The images were captured using a fluorescent microscope.
**RESULTS**

Identification of CRTC2 in the Pin1-containing Complex from Mouse Liver—The adenovirus to MEF-tagged Pin1 was introduced into mice, and the Pin1-containing complex was purified. Purified Pin1 in the complex was electrophoresed and subjected to silver staining, which showed the presence of Pin1 bait proteins and many binding proteins (Fig. 1A). Bands (1), (2), and (3) were identified to be DNA-directed RNA polymerase II A, DNA-directed RNA polymerase IIB, and DNA-directed RNA polymerase I by the analysis using LC/MS, which agree with previous reports (23). Then we performed the immunoblotting using many antibodies to detect another protein included in the Pin1-containing complex because many faint bands were visible with silver staining.

Many transcriptional co-activators are included among the target proteins of Pin1 (4, 5). In addition, although one of the regulatory mechanisms of Pin1 is protein stabilization, recent reports have shown that Pin1 is involved in translocation of target proteins, such as Bax (24). These results suggest that CRTC2 is a candidate Pin1 target protein because CRTC2 is a transcriptional co-activator and is translocated between the cytosol and the nucleus. As a result, immunoblotting using anti-CRTC2 antibody indicated the presence of CTRC2 in the Pin1 complexes (Fig. 1B). To confirm the association between CTRC2 and Pin1, CRTC2 and each GFP-Pin1 or GFP were simultaneously overexpressed in HepG2 and Sf9 cells. As shown in Fig. 1C and supplemental Fig. 1, GFP-Pin1, but not GFP alone was detected in the anti-GFP-Pin1, but not GFP alone was detected in the anti-CRTC2 immunoprecipitate. Furthermore, CRTC2 was detected in the immunoprecipitate with anti-Pin1 antibody but not that with the control IgG from mouse liver (Fig. 1D). Thus, the association between CRTC2 and Pin1 is physiological.

Pin1 possesses the WW and PPlase domains in its N terminus (amino acids 1–38) and C terminus (amino acids 39–163), respectively. To identify the domain of Pin1 responsible for the association with CRTC2, we prepared GST-Pin1, the GST-Pin1 WW domain, and the GST-Pin1 PPlase domain. These GST proteins were conjugated to beads, followed by incubation with cell lysates from MEF-tagged CRTC2 overexpressing HepG2 cells. GST-Pin1 but not GST alone bound to CRTC2 in vitro (Fig. 1E). Using this pull-down system, it was shown that the GST-WW domain, but not the GST-PPlase domain, binds to CRTC2 (Fig. 1F). In addition, okadaic acid treatment significantly increased the association of CRTC2 with Pin1 (Fig. 1G),
suggesting the involvement of serine and/or threonine phosphorylation(s) in CRTC2.

**Pin1 Associates with Ser136-containing Motif in the NLS Domain of CRTC2**—Subsequently, to reveal the domain of CRTC2 responsible for the association with Pin1, six Ds-Red-tagged CRTC2 N terminus deletion mutants (Fig. 2A) and GFP-tagged Pin1 were simultaneously overexpressed in Sf9 cells. As shown in Fig. 2B, CRTC2 deletion mutant 2 (D-2), containing amino acids 121–238, was immunoprecipitated with GFP-tagged Pin1 but not with GFP alone. This portion contains three serine-proline motifs (Fig. 2C). Each of these serine residues was replaced with alanine, creating a mutant that did not associate with Pin1. As shown in Fig. 2D, CRTC2 with serine 136 replaced by alanine did not bind to Pin1, whereas CRTC with serine 129 or 131 bound to Pin1 (data not shown). These observations indicated that the association between CRTC2 and Pin1 is mediated via the phosphoserine 136-containing motif in CRTC2 and the WW domain in Pin1. Ser136 is in the NLS domain, and a high level of Ser136 phosphorylation was demonstrated in a previous report (16).

**Pin1 Inhibits CRTC2 Translocation from the Cytosol to the Nucleus**—To test whether or not the effect of Pin1 on CRE transcripational activity is mediated via the effect on the subcellular localization of CRTC2, the GFP-tagged CRTC2 and Pin1 was mediated via the phosphoserine 136-containing motif in CRTC2 and the WW domain in Pin1. Ser136 is in the NLS domain, and a high level of Ser136 phosphorylation was demonstrated in a previous report (16). In the control LacZ-overexpressing or control siRNA-treated HepG2 cells, GFP-tagged CRTC2 was translocated from the cytosol to the nucleus, as reported previously (9). Pin1 overexpression markedly inhibited forskolin-induced translocation of CRTC2 into the nucleus. In addition, gene silencing of Pin1 using siRNA markedly enhanced the nuclear translocation of Pin1 in comparison with treatment with control siRNA. Although nuclear CRTC2 S136A (unable to bind to Pin1) was required for forskolin stimulation, it had no effect on either Pin1 overexpression or Pin1 siRNA (Fig. 3B).

In addition, we investigated the effect of Pin1 on the distribution of CRTC2 S171A. CRTC2 S171A (unable to bind to 14-3-3) was mainly present in the nucleus regardless of forskolin stimulation (supplemental Fig. 2). Pin1 overexpression slightly increased CRTC2 S171A in the cytosol, whereas Pin1 siRNA treatment reduced the amount of CRTC2 S171A in the cytosol. This effect of Pin1 was essentially in agreement with the results obtained for wild-type CRTC2. Similar results were obtained by immunostaining the endogenous CRTC2 in HepG2 cells (Fig. 3C). Pin1 overexpression attenuated the forskolin-induced nuclear translocation of CRTC2 as compared with LacZ overexpression. On the other hand, treatment with Pin1 siRNA increased CRTC2 in the nucleus under forskolin stimulation as compared with the control siRNA.
Neither the distribution nor the expression of Pin1 was changed by forskolin or insulin stimulation (supplemental Fig. 3). Thus, a change in Pin1 is not required for regulation of the CRTC2 distribution.

**Pin1 Associates with CRTC1 and Induces Its Localization in the Cytosol**—The CRTC family consists of three isoforms, CRTC1, CRTC2, and CRTC3. The motif of CRTC2 responsible for the association with Pin1 is present in the NLS and is conserved in CRTC1 but not in CRTC3 (supplemental Fig. 4A). Thus, the associations of Pin1 with CRTC1 were also investigated using HepG2 cells. As shown in supplemental Fig. 4B, FLAG-tagged CRTC1 was detected in anti-GFP immunoprecipitates from the cells expressing GFP-tagged Pin1 and FLAG-tagged CRTC1. As shown in supplemental Fig. 4C, FLAG-tagged CRTC1, in which serine 155 is replaced with alanine, did not bind to GFP-tagged Pin1, unlike the FLAG-tagged wild-type CRTC1.

Then the effects of Pin1 on localizations of CRTC1 were examined. When LacZ was overexpressed, GFP-tagged CRTC1 was present in the cytosol and translocated to the nucleus in response to forskolin stimulation (supplemental Fig. 4D). This translocation was markedly inhibited by Pin1 overexpression (supplemental Fig. 4D).

**CRTC2 Associated with Pin1 Did Not Bind to CREB**—Formation of the CREB-CBP-CTRC complex, which binds to a CRE site, is critical for CRE transcriptional activation. We investigated whether or not the CREB-CBP-CTRC-Pin1 complex can form, using the baculovirus and S9 cell overexpression system. When CRTC2 and CREB were both overexpressed in HepG2 or S9 cells, CREB was detected in the CRTC2 immunoprecipitate. Interestingly, the overexpression of Pin1 markedly reduced the association between CREB and CRTC2, in either HepG2 or S9 cells (Fig. 4, A and B).

Furthermore, the effect of Pin1 on the association between CRTC2 and 14-3-3 was investigated. In S9 cell lysates overexpressing CREB and CRTC2, both CRTC2 and endogenously expressed 14-3-3 protein were detected in anti-CREB immunoprecipitates (Fig. 4C). In the case of triple overexpressions of CRTC2, CREB, and GFP-tagged Pin1, CRTC2 and 14-3-3 were detectable in the GFP-tagged Pin1 immunoprecipitate (Fig. 4D).

Similar results were obtained in the HepG2 cells. The association between MEF-tagged CRTC2 and endogenously expressed 14-3-3 was not affected by the overexpression of Pin1 (supplemental Fig. 5A). In addition, Pin1 overexpression did not affect the phosphorylation level of Ser171, responsible for the association with 14-3-3, in either basal or forskolin-stimulated conditions (supplemental Fig. 5B). These results suggest that Pin1-associated CRTC2 is capable of binding to 14-3-3 protein but not to CREB.

**Pin1 Inhibits CRE Transcriptional Activity and Its Downstream PEPCK Expression**—Subsequently, to elucidate the role of Pin1 in CRE transcriptional activity, the effects of Pin1 overexpression and Pin1 gene silencing using siRNA on the CRE and PEPCK luciferase assay, and PEPCK mRNA level were investigated in HepG2 cells (Fig. 5). The amount of overexpressed Pin1 was ~5 times that of endogenous Pin1 in HepG2 cells. Under these conditions, forskolin-induced transcriptional activity and PEPCK mRNA induction were significantly attenuated (Fig. 5, A–C). On the contrary, gene suppression of Pin1 using siRNA significantly enhanced these events (Fig. 5, D–F). In addition, suppressions of CRE-luciferase and PEPCK-luciferase activities by Pin1 overexpression were observed in immortalized human hepatocytes (supplemental Fig. 6) (25), suggesting that this mechanism is independent of the glucose sensitivity of the cell type. An inhibitory effect of Pin1 on CRE luciferase activity was observed when wild type or S171A CRTC2, but not S136A, was overexpressed, consistent with the results showing Pin1 to regulate the translocation of CRTC2 (supplemental Fig. 7). Thus, the Pin1 expression level was revealed to negatively regulate CRE transcriptional activity.

**Chromatin Immunoprecipitation Assay with Anti-CRTC2 and CREB Antibodies**—Because Pin1-associated CRTC2 did not bind CREB, we performed a ChiP assay to investigate whether or not Pin1 affected recruitment of CRTC2 to cAMP-responsive elements upstream of PEPCK, NR4A2, and CGA genes (Fig. 5G). The PCR product of the anti-CRTC2 immunoprecipitate was unchanged regardless of forskolin stimulation or Pin1 overexpression. In contrast, the PCR product of the anti-CRTC2 immunoprecipitate was markedly increased by forskolin stimulation, and Pin1 overexpression abolished this increase. Forskolin stimulation induced CBP recruitment to the promoter as well as CRTC2, but Pin1 overexpression had no effect.
Thus, it was suggested that CRTC2 associated with Pin1 was removed from CREB located in the CRE sequence in the PEPCK, NR4A2, and CGA promoter region.

Hepatic Pin1 Overexpression Reduces PEPCK Expression and Decreases Hyperglycemia in STZ-induced Diabetic Mice—CRTC2 is a major transcriptional co-activator for hepatic glucose regulation via its effects on PEPCK expression. Thus, we considered the possibility of the regulation of PEPCK expression by Pin1 in the liver, and an adenovirus expressing Pin1 was introduced into STZ-induced insulin-deficient diabetic mice. Due to the insulin deficiency, as reported previously, hepatic PEPCK mRNA and serum blood glucose levels were markedly increased in fed and fasted state, as compared with the control mice (Fig. 6). The adenovirus for Pin1 expression was injected intravenously, and 96 h later, overexpressed Pin1 was detected only in the liver (Fig. 6A) and not in other tissues. With Pin1 overexpression in the liver, the increased hepatic PEPCK mRNA level in STZ-mice was normalized, and blood glucose elevation was also partially but significantly reduced in both the fed and the fasting state (Fig. 6, B–E). Pin1 overexpression exerted the same effects on other CRE-dependent transcriptional genes, such as G6Pase, PGC-1α, and CPT-1. These findings revealed Pin1 to be a regulator of CRE-dependent transcriptional genes in vivo.

Pin1 Expression Is Low in Fasting State—Finally, we investigated the changes in Pin1 expressions under different nutrient condi-

FIGURE 5. Pin1 suppresses CRE luciferase activity and PEPCK mRNA level in HepG2 cells. A and B, LacZ or Pin1 was overexpressed in HepG2 cells transfected with pTAL and pTAL-CRE or pTAL-PEPCK. D and E, these transfected HepG2 cells were treated with control siRNA or Pin1 siRNA. In two experiments, with and without forskolin stimulation for 6 h, the cell lysates from HepG2 cells were subjected to the luciferase assay. C and F, PEPCK mRNA levels were also measured. Representative data from four independent experiments are shown. **, p < 0.01 versus LacZ or negative siRNA. G, HepG2 cells overexpressing LacZ or Pin1 were subjected to the CHIP assay using anti-CRTC2, anti-CNP, or anti-CREB antibodies and primers corresponding to the PEPCK, NR4A2, and CGA promoter regions. Representative data from four independent experiments are shown. IB, immunoblot; IP, immunoprecipitation. Error bars, S.E.
Interestingly, we found that the Pin1 expression level is low in the fasted state but is increased by feeding (Fig. 7). Thus, Pin1 expression appears to be regulated by nutrient conditions.

**DISCUSSION**

CRE transcriptional activity is enhanced through association of the CREB-CBP-CRTC complex on a CRE site. The co-activator of CREB termed the CRTC family consists of three isoforms, CRTC1, CRTC2, and CRTC3 (18). CRTC2 was reported to be important for the regulation of CRE transcriptional activity and its downstream PEPCK gene expression (20). Depletion of nuclear CRTC2 leads to the suppression of CRE transcriptional activity (20). Thus, both the subcellular localization of CRTC2 and CREB-CBP-CRTC complex formation are critical for CRE transcriptional activity. CRTC2 is reportedly phosphorylated by AMPK and SIK, and phosphorylated CRTC2 binds to 14-3-3 protein and is thereby shifted from the nucleus to the cytoplasm (21). The Montiminy group (16) has identified 12 independent phosphorylated serine residues on CRTC2 using tandem MS analysis. They demonstrated that PKA inhibits the activity of SIK and reduces Ser171 phosphorylation leading to binding with 14-3-3 protein and translocation to the cytosol (16). However, the importance of other phosphorylation sites identified in their study, such as Ser136, remains unknown.

**Figure 6.** Hepatic overexpression of Pin1 restored elevated CRE-dependent transcriptional genes and hyperglycemia in STZ-treated mice. STZ-treated diabetic C57BL/6 male mice were injected with 2.5 × 10⁷ plaque-forming units/g body weight of adenovirus containing β-galactosidase (LacZ) or FLAG-tagged Pin1 construct via the tail vein. A, immunoblotting of hepatic tissue lysates with anti-FLAG or anti-Pin1 antibody. B and C, serum glucose concentrations in fed and fasting states (n = 6, each group). D and E, CRE-dependent transcriptional gene mRNA levels in the liver. **, p < 0.01 versus STZ; ***, p < 0.001 versus STZ. Error bars, S.E.

**Figure 7.** Pin1 expression is regulated by nutrient conditions. Mice were fed routinely, starved for 20 h, or refed for 4 h after a 20-h fast. Liver (left) and muscle (right) cell lysates were prepared and then immunoblotted with anti-Pin1 antibody. A representative immunoblot (IB) is shown in the upper panel.
Pin1 expression level is involved in regulating glucose metabolism in STZ-treated mice suggests that the reduced high PEPCK expression and its resultant fasting effect of glucagon. The fact that Pin1 overexpression controls CRE transcriptional activity are reportedly up-regulated in these mice, gluconeogenic enzymes, such as PEPCK, under the hyperglycemia in insulin-deficient STZ-treated mice. In fact, our observations using GFP-tagged CRTC1 and CRTC2 as well as staining of endogenous CRTC2 supported our hypothesis. On the other hand, gene silencing of Pin1 using siRNA markedly induced nuclear localization of CRTC2 when stimulated with forskolin. It is likely that altered localization of CRTC2 due to Pin1 takes place independently of the binding of 14-3-3 protein to CRTC2 because Pin1 overexpression affected neither the Ser\textsuperscript{171} phosphorylation level of CRTC2 nor the association with 14-3-3.

A further interesting issue is that CRTC2 associated with Pin1 did not bind to CREB. This phenomenon cannot be attributable to the different subcellular distributions of CREB, CBP, and CRTC because highly overexpressed CREB, CBP, and CRTC2 are present in the cytosol of Sf9 cells. Taken together, these observations indicate the association of Pin1 with CRTC2 to decrease the nuclear CBP-CRTC2-CREB complex via two mechanisms (i.e. the export of CRTC2 and interruption of the association between CRTC2 and CREB). Thus, the Pin1 expression level is a key factor regulating CRE transcriptional activity.

We investigated the effects of various kinase inhibitors on the association between CRTC2 and Pin1, using HepG2 cells, in an effort to identify the kinase that is involved in the phosphorylation of S136A on CRTC2. However, we were unable to obtain clear results. Although we did not discover which kinase(s) phosphorylates the Ser\textsuperscript{136} of CRTC2 responsible for the association with Pin1 in this study, high basal phosphorylation of Ser\textsuperscript{136} was already demonstrated in a previous report (16). Prior studies have also shown that Pin1 expression generally correlates with cell proliferative potential in normal tissues (1, 26, 27) and is further up-regulated in many human cancers (28–31). In addition, interestingly, we noticed that the amount of Pin1 was higher in the fed than in the fasting state, in both liver and muscle. However, neither insulin nor forskolin has any effect on the expression of Pin1 in HepG2. Thus, the mechanism(s) involved in the altered expression of Pin1 remains unclear, although this is an important issue that merits further investigation.

In the liver, CRE transcriptional activity plays a critical role in gluconeogenesis (32–34). In addition, in the diabetic state, insufficient suppression of CRE transcriptional activity is regarded as a mechanism underlying hyperglycemia under fasted conditions (35). In the present study, our final experiment examined whether Pin1 overexpression might improve the hyperglycemia in insulin-deficient STZ-treated mice. In these mice, gluconeogenic enzymes, such as PEPCK, under the control of CRE transcriptional activity are reportedly up-regulated (20, 36, 37) due to insulin deficiency and the relatively increased effect of glucagon. The fact that Pin1 overexpression reduced the high PEPCK expression and its resultant fasting serum glucose elevation in STZ-treated mice suggests that the Pin1 expression level is involved in regulating glucose metabolism. Thus, an agent affecting Pin1 expression or activity may represent a novel therapeutic strategy for diabetes.

To date, numerous proteins have been identified as substrates of Pin1 (4, 5, 38). With the proline conformational change induced by Pin1, the structure and function of the target protein are modified, which affects protein stabilization, subcellular localization, phosphorylation, transcriptional activity, etc. In the case of CRTC2, both subcellular localization and the complex-forming function with CREB are affected.

Although we did not investigate the physiological effects occurring via CRTC1 induced by the association with Pin1, we did observe that Pin1 is highly expressed in the brain, whereas its enzymatic activity is blunted by oxidative stress modification that occurs in the early stages of Alzheimer disease (39). Although the physiological function of Pin1 in neurons remains largely unknown, numerous reports have implicated CRE transcriptional activity in brain function (40–42). Thus, further important evidence may be obtained from studies of Pin1 and CRTC1 in the brain or other tissues.

In summary, CRTC2 was identified as a new Pin1-binding protein. The CBP-CRTC2-CREB complex promotes gluconeogenesis. Pin1 binding to CRTC2 prevents this complex formation, thereby suppressing CRE transcriptional activity (supplemental Fig. 8). These findings indicate that Pin1 is a regulator of gluconeogenesis and may be a new target for diabetic therapy.

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