Nuclear translocation of CBP/p300-interacting protein CITED1 induced by parathyroid hormone requires serine phosphorylation at position 79 in its 63–84 domain

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The transcriptional cofactor CITED1 inhibits osteoblastic differentiation and blunts the stimulation of osteoblastic differentiation by parathyroid hormone (PTH). In the MC3T3-E1 osteoblastic cell line, we found that CITED1 was located predominantly in the cytoplasm and that hPTH(1–34) increased translocation of CITED1 from the cytoplasm to the nucleus. This response to hPTH(1–34) was not observed when all 9 serine residues within the 63–84 domain of CITED1 were mutated to alanines (CITED1 S9A) or when a single serine to alanine mutation was made at position 79 (CITED1 S79A). CITED1 containing mutations of these 9 serines to glutamic acid (S9E) retained the same nuclear translocation response to hPTH(1–34) as the wild type CITED1. ALP activity and formation of mineralized nodules were inhibited in cells transfected with pcDNA3-CFP-CITED1 or with pcDNA3-CFP-CITED1 S9E with or without hPTH(1–34) treatment (all P < 0.05); these changes were not observed using CITED1 S9A. Cells exposed to intermittent treatment with hPTH(1–34) expressed more ALP, Runx2 and osteocalcin than vehicle-treated cells. These effects of hPTH(1–34) were inhibited in cells transfected with pcDNA3-CFP-CITED1 or pcDNA3-CFP-CITED1 S9E, but were slightly enhanced by the alanine mutants. PKC activator (TPA) increased nuclear translocation of CITED1, whereas a PKC inhibitor (Go6983) blunted the effect of hPTH(1–34) on the nuclear translocation of wildtype CITED1 but not of CITED1 S79E. The data indicated that serine phosphorylation at position 79 in the 63–84 domain is associated with PKC activation, and is required for both CITED1 nuclear translocation induced by PTH and the negative effects of CITED1 on osteoblastic differentiation and mineralization.

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1. Introduction

Parathyroid hormone (PTH)(1–34) is one of the most important factors known to promote osteoblastic bone formation via recruitment of local growth factors and stimulation of osteoblastic differentiation and anti-apoptotic activities [1,2]. Intermittent administration of hPTH(1–34) has been shown to increase bone mineral density and to improve the mechanical strength of bone [3]. These effects are partly mediated through the PTH-Smads3 axis [4], the Wnt/β-catenin pathway [5], the RANKL pathway [6], and cAMP signaling [7,8]. However, these pathways do not explain the entire response of osteoblasts to PTH. CBP/p300 interacting transactivator with glutamic acid and aspartic acid rich C terminal domain 1 (CITED1), formerly named melanoma specific growth gene 1 (msg-1), is a transcriptional cofactor originally found in a murine melanoma cell line [9,10]. CITED1 enhances Transforming Growth Factor (TGF)-β induced transcription via the interaction of its conserved C-terminal transcriptional activating domain (CR2 domain) with CBP/p300 to form a complex [9,11]. CITED1 also selectively binds the activation function 2 domain of estrogen receptors and enhances estrogen-dependent gene transcription [12]. In one of our previous studies, CITED1 was found to be expressed in osteoblasts, and its expression was increased by PTH(1–34). Deletion of the CITED1 gene increased osteoblastic differentiation in vitro, and CITED1 was identified as a negative feedback regulator in the positive effect of PTH on osteoblastic differentiation [13].

CITED1 has been reported to localize in the cytoplasm of kidney cells [9,14], as expected based on its function as a transcriptional cofactor, but...
nuclear accumulation has also been described in Wilm’s tumor cells [15]. The translocation of CITED1 from nuclei to cytoplasm has been associated with the 158LMSLVVELGL167 sequence in its CR2 domain, but the determinant(s) of the translocation of CITED1 from the cytoplasm to the nucleus, which could be the key regulatory point for CITED1’s function, is still unknown [16]. In a recent study, we observed that PTH(1–34) induced CITED1 translocation from the cytoplasm into the nuclei in MC3T3-E1 osteoblastic cells, which suggests that CITED1 may play a role in regulating gene transcription induced by PTH.

Serine phosphorylation has been associated with CITED1 binding to p300 and its transactivation functions [16]. It is also an important mechanism for the action of protein kinases downstream of PTH signaling. We proposed that PTH may alter the phosphorylation status of key serine residues in the CITED1 molecule to increase its nuclear translocation. In this study, we aimed to investigate the possible effect of serine phosphorylation in CITED1 on its nuclear translocation and thereby its regulation of osteoblastic differentiation.

2. Materials and methods

2.1. Peptide preparation

Human PTH(1–34) (hPTH(1–34)) was synthesized and quantified by amino acid analysis at the Biopolymer Core Laboratory of the Endocrine Unit, Massachusetts General Hospital (Boston, MA, USA). The peptide was diluted and stored in 0.1% trifluoroacetic acid (TFA) at −20 °C.

2.2. Cell culture

Mouse osteoblastic MC3T3-E1 subclone 14 (ATCC CRL-2594), purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in a modified α-MEM culture medium (HyClone, Thermo Fisher Scientific, Waltham, MA, USA), containing 10% fetal bovine serum (FBS) (GIBCO, Invitrogen Inc., Carlsbad, CA, USA) and 1% penicillin/streptomycin (GIBCO, Invitrogen Inc., Carlsbad, CA, USA) in a 5% CO2 humidified atmosphere.

Primary calvarial osteoblasts were used to be a source of CITED1 cDNA were isolated from the calvariae of C57BL mouse neonates. All procedures and animal experiments were approved by the Nanfang Hospital of Southern Medical University (Guangzhou, China) and conducted in accordance with all state regulations. In brief, one-day-old C57BL mice were purchased from the animal facility of the Nanfang Hospital. Frontoparietal bones from neonatal mice were dissected and minced, and then were subjected to sequential collagenase digestion (types I and II; ratio: 1:3; Sigma-Aldrich, St Louis, MO, USA). Of the six fractions generated by serial 20 min digests, fractions 3–6 were combined, and the cells were plated at 5 × 10⁴ cells/cm² in a humidified atmosphere (95% air–5% CO2) in αMEM supplemented with 10% FBS [17]. Primary calvarial osteoblasts were greater than 70%.

2.3. CITED vector construction

Total RNA from primary calvarial osteoblasts was extracted (Omega Bio-tek, GA, USA) and reversely transcribed to cDNA (Takara, Dalian, China), from which the CITED1 gene was cloned using designed primers (forward: 5’-GAT CCG TAC CCG AAC CAT GAC GGC GGC TGC A-3’; reverse: 5’-GAT CCG CCC TCA GCA GCA AGG AAA -3’), crosslinked with cyan fluorescent protein (CFP) (Invitrogen, NY, USA), and then inserted into pcDNA3 vector containing Kpn I and Not I (Invitrogen, NY, USA) (pcDNA3-CFP-CITED1). Mutated CITED1 DNA sequences (9S–A or 9E–E) were artificially synthesized (Generay Biotech Co., Ltd, Shanghai, China), inserted into pcDNA3 vector (pcDNA3-CFP-CITED1 9S–A, pcDNA3-CFP-CITED1 9S–E, pcDNA3-CFP-CITED1 9E–A, pcDNA3-CFP-CITED1 9E–E) and verified by DNA sequencing.

2.4. Transient transfection

MC3T3-E1 cells were plated into confocal dishes (Coldspring Science Corporation, Fairfax, VA, USA) at a density of 5 × 10⁴ cells/well, into cell culture plates (Corning Inc., Corning, NY, USA) at 3 × 10⁵ cells/well (24-well plates) or 3 × 10⁶ cells/well (6-well plates), or into 4-well culture slides (BD Falcon, Bedford, MA, USA) at 2 × 10⁶ cells/well. Then, cells were transfected using the Fugene 6 transfection reagent (Roche Molecular Systems, Pleasanton, CA, USA) and the above plasmids (0.25 μg DNA/cm² of bottom surface area) using a ratio of 1 μg of DNA to 3 μl of Fugene 6, according to the manufacturer’s instructions. Transfections were performed in FBS-free medium for 4 h, and FBS was then added to a final concentration of 10%.

2.5. Immunofluorescence and confocal microscopy

48 h after MC3T3-E1 cells were plated into 4-well culture slides, the cells were switched into a medium containing α-MEM and 1% FBS for 12 h; the medium was then replaced with Hank’s solution + α-MEM (ratio = 8:2) and cultured for another 20 min. Then, 100 nM of hPTH(1–34) or vehicle (0.1% TFA) was added to the cells. After 30 min or 2 h, cells were rinsed gently with PBS and fixed in 4% paraformaldehyde for 30 min. Attached cells were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 min, followed by blocking for 30 min in 5% bovine serum albumin (BSA, Mostbruck Chemicals Co., Ltd., Shanghai, China) and incubation with a goat polyclonal antibody against CITED1 (SC-50743, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight, followed by addition of Texas red-coated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, cells were washed in PBS and stained with 2 μg/ml 4–6-diamidino-2-phenylindole (DAPI) (Beyotime Institute of Biotechnology, Jiangsu, China), according to the manufacturer’s instructions. Signals were visualized by confocal fluorescence microscopy (FV10i-W, Olympus, Tokyo, Japan). For subcellular localization studies in live cells, the constructs of pcDNA3-CFP-CITED1, pcDNA3-CFP-CITED1 9S–A, pcDNA3-CFP-CITED1 9S–E, pcDNA3-CFP-CITED1 9E–A or pcDNA3-CFP-CITED1 9E–E were transiently transfected into MC3T3-E1 cells cultured on confocal dishes, and live cells were visualized using an inverted confocal microscope (TCS SP2 A08S, Leica Microsystems, Wetzlar, Germany).

2.6. Cell differentiation and alkaline phosphatase activity

Cells were plated in 24-well plates coated with type 1 collagen at 3 × 10⁴ cells/well, and incubated at 37 °C. They were then transfected with pcDNA3-CFP-CITED1, pcDNA3-CFP-CITED1 9S–A, pcDNA3-CFP-CITED1 9S–E, pcDNA3-CFP-CITED1 9E–A or pcDNA3-CFP-CITED1 9E–E. After 3 days, the medium was changed to a mineralization medium of α-MEM supplemented with 10% FBS, 100 mM l-glycerophosphate, 50 μg/ml ascorbic acid and 100 nmol/L dexamethasone. Intermittent treatment with hPTH(1–34) was carried out based on 4/48 h cycles, as previously described [13]. In brief, cells were cultured with 10 nM of hPTH(1–34) for 4 h, and then switched to a fresh medium without hPTH(1–34) for the following 44 h. At week 4, cultured cells were fixed with 4% paraformaldehyde for 30 min, rinsed three times with PBS, and then treated with BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Institute of Biotechnology, Haimen, China) for 30 min to demonstrate alkaline phosphatase (ALP) expression. Proteins from each well was extracted with 0.2% Triton X-100, and ALP activity was measured by incubating cell lysates in ALP substrate buffer (Jiancheng Bioengineering Institute, Nanjing, China) containing the soluble substrate p-nitrophenyl.
phosphate, according to the manufacturer’s instruction. The activity was determined as the absorbance at 520 nm. A standard curve was prepared with p-nitrophenol (Jiancheng Bioengineering Institute, Nanjing, China). Each value was normalized to the total protein content measured with a BCA protein measure kit (Jiancheng Bioengineering Institute, Nanjing, China).

2.7. Mineralization assay

For analysis of matrix mineralization, cells were plated, treated and fixed as above, and the presence of mineralized nodules was assessed using Alizarin Red staining. Briefly, the fixed cells were rinsed with distilled water three times, followed by incubation with 1% Alizarin Red S.
solution (Sigma, St Louis, MO, USA) for 30 min at room temperature. After removing excess dye, cells were washed three times with distilled water. Alternatively, to determine the calcium content of the cultures, cell monolayers were washed in Ca^{2+}− and Mg^{2+}-free PBS, and then incubated for 3 h in 0.2 ml of 0.6 N HCl. Extracted calcium was then measured spectrophotometrically at 610 nm after reaction with methylthymol blue (Hui Cheng Biological Technology Co. Ltd, Shanghai, China) [18]. The calcium concentration in each well was normalized to the total protein concentration measured using BCA protein measure kit.

2.8. Real time RT-PCR

MC3T3-E1 cells were plated in type I collagen-coated 6-well plates at a density of 3 × 10^5 cells/well and cultured for 24 h. Then the plasmid transfection and intermittent treatment of hPTH(1–34) were performed as described in Section 2.7. 4 h after the last addition of 10 nM hPTH(1–34), total RNA was isolated from the cells using the TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA), according to the manufacturer’s instructions. RNA purity was determined using the absorbance at 260 and 280 nm (A260/A280). Total RNA was reverse-transcribed into cDNAs using a fQiithera Fisher Scientific, Waltham, MA, USA). Relative mRNA levels were evaluated by quantitative PCR using SYBR® Premix Ex Taq (Takara Bio, Otsu, Japan) in the MX3005P Real-Time QPCR System (Strategene, Wilmington, DE, USA). We quantified osteoblastic differentiation marker genes, such as ALP2 (forward: 5′-GCT GAT ATG AGA TGT CCT T-3′, reverse: 5′-GCA TGCTG CTC TGT CCT ACT-3′), Runx2 (forward: 5′-CCG AGG AGC CAC TTA CTA CA-3′, reverse: 5′-TAT GGA GTC GTG CTC TAC TG-3′), and osteocalcin (OC) (forward: 5′-AAG CAC GAG GGC AAT AAG G-3′, reverse: 5′-GGC GTC TTC AAG CCA TAC TG-3′), osteocalcin (OC) (forward: 5′-AAG CAC GAG GGC AAT AAG G-3′, reverse: 5′-GGC GTC TTC AAG CCA TAC TG-3′), and osteocalcin (OC) (forward: 5′-AAG CAC GAG GGC AAT AAG G-3′, reverse: 5′-GGC GTC TTC AAG CCA TAC TG-3′), and osteocalcin (OC) (forward: 5′-AAG CAC GAG GGC AAT AAG G-3′, reverse: 5′-GGC GTC TTC AAG CCA TAC TG-3′), and osteocalcin (OC) (forward: 5′-AAG CAC GAG GGC AAT AAG G-3′, reverse: 5′-GGC GTC TTC AAG CCA TAC TG-3′), and osteocalcin (OC) (forward: 5′-AAG CAC GAG GGC AAT AAG G-3′, reverse: 5′-GGC GTC TTC AAG CCA TAC TG-3′), and osteocalcin (OC) (forward: 5′-AAG CAC GAG GGC AAT AAG G-3′, reverse: 5′-GGC GTC TTC AAG CCA TAC TG-3′), and osteocalcin (OC) (forward: 5′-AAG CAC GAG GGC AAT AAG G-3′). The housekeeping gene GAPDH (forward: 5′-TGT CCTG GCA GTC TAC TGG TG-3′, reverse: 5′-GCA CTG CCA CTG CCT ACT-3′) was used as the internal control. Reactions were performed using the following thermal conditions: initial denaturation for one cycle at 95 °C for 3 s, annealing and extension at 60 °C for 34 s. First, ΔΔCt was calculated as Ct target gene − Ct GAPDH. Then, ΔΔCt was calculated as ΔCt treated − ΔCt control. Lastly, 2^−ΔΔCt was calculated to represent the relative mRNA expression of target genes.

2.9. Statistical analysis

All statistical analyses were conducted using SPSS version 13.0 (SPSS Inc., Chicago, IL). Data are expressed as means ± standard deviation (SD). Variables were analyzed using analysis of variance (ANOVA) and Bonferroni’s test for post hoc analysis. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. hPTH(1–34) induces nuclear translocation of CITED1

Our previous study showed that hPTH(1–34) enhances the expression of CITED1 [13]. After 100 nM of hPTH(1–34) was added into the culture medium, we observed a progressive movement of fluorescent CFP-CITED1 from the cytoplasm into the nuclei, starting at 5 min after addition up to 30 min (Fig. 1A). To validate whether this response was endogenously occurring in osteoblasts, we detected expression of CITED1 protein in hPTH(1–34)-naive and hPTH(1–34)-treated MC3T3-E1 cells using a CITED1-specific antibody. Results showed that with vehicle only for 2 h and 100 nM of hPTH(1–34) for 30 min and 2 h. Without hPTH(1–34) treatment, most of the CITED1 remained in the cytoplasm. However, after 30 min of incubation with 100 nM of hPTH(1–34), the nuclear translocation of CITED1 was visible, and after 2 h of incubation, most of the CITED1 had translocated to the nucleus (Fig. 1B).

3.2. S-A mutations in the 63–84 domain of CITED1 abolish its nuclear translocation

We used the NetPhos 2.0 Server software (http://www.cbs.dtu.dk/services/NetPhos/) to identify the possible phosphorylation sites of serines in CITED1. We found that in the 63–84 region of the protein, 8 of 9 serine residues had high scores for phosphorylation (the 63rd, 64th, 67th, 69th, 73rd, 79th, 80th and 84th residues; but not the 65th) (Table 1). We then mutated all 9 of these serines into alanines to eliminate the 63–84 domain are essential for hPTH(1–34)-induced CITED1 translocation.

Table 1 Identification of possible phosphorylation sites on CITED1.

<table>
<thead>
<tr>
<th>Position in protein sequence</th>
<th>Sequence</th>
<th>Phosphorylation score</th>
<th>Kinase (score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>MPTM</td>
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<td>CKII (0.57)</td>
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<td>DNAK (0.51)</td>
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<tr>
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<td>CKI (0.51)</td>
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<td>CKII (0.64)</td>
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<tr>
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<td>ADHP</td>
<td>0.051</td>
<td>CKII (0.50)</td>
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Fig. 2. S–A or S–E mutation in region 63–84 affected nuclear translocation of CITED1. MC3T3-E1 cells were plated into confocal dishes at $5 \times 10^3$ cells/well (A, B and C), or into 4-well culture slides (D, E and F) at $2 \times 10^4$ cells/well. After 24 h, cells were transfected with pcDNA3-CFP-CITED1 (A and D), pcDNA3-CFP-CITED1 9S–A (B and E) or pcDNA3-CFP-CITED1 9S–E (C and F) and cultured for 48 h. Thereafter, cells were cultured in α-MEM with 1% FBS for 12 h; the medium was then replaced with Hank’s solution + α-MEM (ratio = 8:2) and cultured for another 20 min. In panels A, B and C, the cells were investigated under the fluorescence microscope at 0 min and 30 min after the addition of 100 nM hPTH(1–34). In panels D, E and F, 30 min or 2 h after 100 nM hPTH(1–34) treatment, cells were fixed and the expression of CITED1 was detected with goat polyclonal antibody against CITED1 and Texas red donkey anti-goat IgG. Nuclei were stained with DAPI (× 600).
3.3. S–E mutations in 63–84 domain of CITED1 maintain its nuclear translocation

We then mutated all 9 serines into glutamates (CITED1 S9–E). When CITED1 S9–E was transfected into MC3T3-E1 cells, hPTH(1–34)-induced nuclear translocation was once again observed (Fig. 2C and F), and was similar to wild-type CITED1 (Fig. 2A and D). Without treatment with hPTH(1–34), CITED1 visibly stayed in the cytoplasm (Fig. 2F), which implies that the phosphorylation of serines in the 63–84 domain is not the only factor involved in CITED1’s nuclear translocation in response to PTH.

3.4. Serine mutations in the 63–84 domain affect CITED1’s inhibition of MC3T3-E1 cell osteoblastic differentiation and mineralization

It has been known that CITED1 inhibits osteoblastic differentiation [13]. To understand whether the phosphorylation in the 63–84 domain of CITED1 had a direct effect on its function, we transiently transfected pcDNA3-CFP-CITED1, pcDNA3-CFP-CITED1 S9–A or pcDNA3-CFP-CITED1 S9–E into MC3T3-E1 cells and compared their effects on osteoblastic differentiation. 4 weeks after 4/48 h cyclic PTH treatment, ALP activity was demonstrated by BCIP/NBT alkaline phosphatase assay. In vehicle-treated cells, MC3T3-E1 cells transfected with pcDNA3-CFP-CITED1 or pcDNA3-CFP-CITED1 S9–E showed decreased ALP activity compared with control group (MC3T3-E1 cells transfected with empty vector) [both P < 0.01], while CITED1 S9–A transfected cells displayed an ALP activity similar to the control group (P > 0.05). After adding PTH 10 nM, ALP activity was increased compared with the vehicle treatment, and the general effects of CITED1 mutations remained the same (Fig. 3A). Osteoblastic mineralization was analyzed by a qualitative assay using Alizarin Red staining, while a quantitative assay was performed based on direct measurement of the calcium content in the cultures. The regulation of mineralization was similar to that of ALP activity (Fig. 3B).

3.5. Serine mutations in the 63–84 domain of CITED1 affect mRNA expression levels of different bone markers

Using the vehicle only, ALP2 mRNA expression was decreased in MC3T3-E1 cells transfected with pcDNA3-CFP-CITED1 or pcDNA3-CFP-CITED1 S9–A, while it was increased in cells transfected with pcDNA3-CFP-CITED1 S9–E, compared with control group (all P < 0.05). PTH treatment dramatically increased ALP2 mRNA expression in all groups, but the same trends were observed with respect to the effects of the CITED1 mutations (Fig. 4A).

In vehicle-treated cells, OC mRNA expression was increased in cells transfected with CITED1 S9–A, compared with control group (P < 0.05). Under PTH treatment, OC mRNA expression in cells transfected with pcDNA3-CFP-CITED1 and pcDNA3-CFP-CITED1 S9–E was decreased compared with control group (both P < 0.05), while CITED1 S9–A had no effect (Fig. 4B).

In vehicle-treated cells, Runx2 mRNA expression was decreased in pcDNA3-CFP-CITED1 and pcDNA3-CFP-CITED1 S9–E transfected cells, compared with control group (both P < 0.05). After PTH treatment, cells transfected with pcDNA3-CFP-CITED1 S9–E had increased Runx2 levels, while cells transfected with pcDNA3-CFP-CITED1 S9–E had decreased levels, compared with control group (both P < 0.05) (Fig. 4C).

3.6. Serine79 phosphorylation is required for CITED1 translocation and associated with protein kinase C

With the NetPhos 2.0 Server software, we then analyzed the possible kinases involved in serine phosphorylation in the region of 63–84 (Table 1) and found that protein kinase C (PKC), the signaling closely downstream of PTH, scored highly at the 67th and 79th serines. We therefore investigated the role of PKC in the translocation of CITED1. As shown in Fig. 5A, the nuclear translocation of CITED1 could be induced by a PKC activator and that induced by hPTH(1–34) was inhibited by Go6983 (Selleck chemicals, USA, dissolved in 1% DMSO and added 1 h previously), a pan PKC inhibitor.

The influence of individual serine residues in the 63–84 domain was demonstrated by single amino acid mutations. Among these, S79–A was the only one that inhibited CITED1 nuclear translocation (Fig. 5B and C, data of other single serine mutations not shown). However, PKC inhibitor did not block the translocation of CITED1 with S79–E mutation (Fig. 5D).

The effect of the Serine79 mutation on osteoblastic differentiation and mineralization was also investigated. CITED1 S79–A had a similar effect as CITED1 S9–A, and the S79–E mutation was the same as wildtype CITED1 or CITED1 S9–E (see Supplementary Fig. 1 and Section 3.4).

4. Discussion

We previously demonstrated that CITED1 is acutely and transiently increased in osteoblastic cells after treatment with hPTH(1–34) [13]. In cells derived from CITED1-knockout mice, we showed that CITED1 is an inhibitor of osteoblastic differentiation, and appears to be one component of an autoregulatory feedback loop that serves to restrain the extent of PTH regulation on osteoblastic function [13]. However, how CITED1 affects osteoblastic differentiation and PTH’s function in bone metabolism is still unclear.

In this present study, we demonstrated that hPTH(1–34) enhanced CITED1 nuclear translocation and that this phenomenon was blocked by serine-to-alanine mutations that would eliminate serine phosphorylation, and was maintained by serine-to-glutamate mutations that would mimic serine phosphorylation in the 63–84 region of the CITED1 protein.

CITED1 has been defined as a transcriptional cofactor, and its function would be expected to depend upon its subcellular localization. In cap mesenchyme cells of the developing kidney and in HEK293 cells with CITED1 transfection, 95% of CITED1 was localized in the cytoplasm and only 5% in the nucleus [16]. In our study, CITED1 was first fused with CFP, inserted into the pcDNA3 vector and transfected into the MC3T3-E1 osteoblastic cell line. Using fluorescence microscopy, we observed that the fluorescent signal moved from the cytoplasm to the nucleus within 30 min of treatment with hPTH(1–34). The response was time-dependent and significant 30 min after PTH addition. The nuclear localization of CITED1 endogenously occurring in osteoblasts was confirmed using a CITED1-specific antibody and a more dramatic response was seen 2 h after PTH addition. The results demonstrated that beside the enhancement of CITED1 gene expression, PTH also increased the nuclear translocation of CITED1.

The functional domains of CITED1 have been mapped out. The CR2 domain (region 145–193) is important for CBP/P300 and estrogen receptor α binding. The 30–60 region is critical for Smad4 binding, and HSC70 has a close association with the 1–145 region [11,12,19]. Our results add new information to understand the regulation of CITED1 function and the potential role of serine phosphorylation in controlling its subcellular localization. It has been reported that serines in positions 16, 63, 67, 71 and 137 are important for CITED1 binding to p300 and inhibited transactivation of Smad4 and p300, but the mutation (to either alanine or glutamine) did not change the localization of CITED1. Although the 156LMSLVVELGL167 sequence in the CR2 domain of CITED1 has been found to mediate CITED1’s translocation from the nucleus to the cytoplasm, the mechanism whereby CITED1 translocates into nuclei is unclear [16]. Because serine phosphorylation is an important mechanism for controlling the function of regulatory proteins, we analyzed the possible phosphorylation site(s) in CITED1 with the NetPhos 2.0 Server software. CITED1 presents a 9-serine cluster in the 63–84 region: four out of nine scored >0.9, two scored >0.8, one scored >0.6 and one >0.5. In order to assess if the phosphorylation of serines in this region affects CITED1 nuclear translocation, we mutated serines to alanines or glutamates. To our surprise, PTH-driven CITED1 nuclear translocation was blocked by alanine mutations and maintained by glutamine mutations. Our results support the theory that the
Figure A: ALP activity (U/g protein) and Calcium (g/g protein) in Vehicle and PTH treated samples with different conditions. The bars represent the mean ± SEM of triplicate samples. *p < 0.05 compared to the control, and △p < 0.05 compared to CITED1 9S>A.

Figure B: ALP activity (U/g protein) and Calcium (g/g protein) in Vehicle and PTH treated samples with different conditions. The bars represent the mean ± SEM of triplicate samples. *p < 0.05 compared to the control, and △p < 0.05 compared to CITED1 9S>A.
phosphorylation of these serine residues was essential but not sufficient for PTH-induced CITED1 nuclear translocation, because no significant CITED1 nuclear translocation was seen without PTH treatment. The mechanism by which PTH phosphorylates CITED1 and how other residues (serine, threonine or tyrosine) outside this 63–84 region contribute to CITED1 relocation should be studied, since these results may be important not only for studying the bones, but also for studying other tissues and diseases, such as thyroid cancer.

The S–A mutation in the 63–84 region strikingly altered the biological functions of CITED1 in osteoblastic cells, eliminating the inhibitory effects of CITED1 on osteoblastic differentiation and mineralization. The S–E mutation, mimicking serine phosphorylation, demonstrated the same effects as the wild-type CITED1, which concurred with the results showing similar nuclear translocations between wild-type CITED1 and the S–E mutant. However, the mRNA expression levels of different bone markers were more complicated. ALP2 gene expression was down-regulated in CITED1 and CITED1 S–E cells, with or without PTH treatment, and the same phenomenon was observed in OC expression in cells treated with hPTH(1–34). Although ALP activity and calcium deposition were comparable in cells transfected with an empty vector and the CITED1 9S–A mutant, we observed a higher ALP gene expression in CITED1 9S–A cells treated with the vehicle or hPTH(1–34), and more OC gene expression in CITED1 9S–A cells in basal condition. Many genes contribute to osteoblastic differentiation [20], and we did not observe any conflicts between the gene expressions and cellular differentiation. In CITED1 and CITED1 9S–E cells, Runx2 gene expression was down-regulated. After hPTH(1–34) treatment, Runx2 mRNA was up-regulated in CITED1 9S–A cells and down-regulated in CITED1 9S–E cells. Because Runx2 gene is expressed in premature osteoblasts and controls osteoblastic differentiation [21], Runx2 is thought to be an important candidate for CITED1’s function, but the mechanisms need to be fully investigated. The slight stimulatory effects of the 9S–A mutant CITED1 on basal or PTH-regulated gene expression seen in our experiments (Fig. 4) could reflect competition of the highly expressed mutant protein with lower levels of functionally suppressive endogenous wild-type CITED1 protein.

With respect to the actions of intermittent hPTH(1–34) exposure on osteoblastic differentiation and function, hPTH(1–34) was previously shown to increase osteoblastic differentiation and to increase CITED1 expression [13]. In our current study, intermittent hPTH(1–34) treatment increased ALP activity and mineralization in MC3T3-E1 cells transfected with empty vector or S–A mutated CITED1 to higher levels than in cells transfected with wild-type CITED1. However, the ratio of PTH–treated to control levels tended to be comparable regardless of the CITED1 type. Taken together with our previously published results, this implied that hPTH(1–34) has the same positive effects in osteoblastic differentiation with or without CITED1 expression.

5. Conclusion

Nuclear translocation is important for CITED1’s role as a transcriptional cofactor. Results showed that hPTH(1–34) enhanced the nuclear translocation of CITED1, and that this phenomenon was associated with aserine at position 79 in the 63–84 region and PKC activation, which was important for the effects of CITED1 on osteoblastic differentiation. However, the mechanisms underlying CITED1’s nuclear translocation and its relationship with hPTH(1–34) still require further study.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2014.06.015.
Fig. 5. Serine at position 79 was required for CITED1 nuclear translocation and associated with PKC activation. MC3T3-E1 cells were plated into 4-well culture slides at $2 \times 10^4$ cells/well. After 24 h, cells were transfected with pcDNA3-CFP-CITED1 (A), pcDNA3-CFP-CITED1 $S^{79}>A$ (B), pcDNA3-CFP-CITED1 $S^{79}>E$ (C) or pcDNA3-CFP-CITED1 $S^{67}>E$ (D) and cultured for 48 h. Thereafter, cells were cultured in $\alpha$-MEM with 1% FBS for 12 h; the medium was then replaced with Hank’s solution + $\alpha$-MEM (ratio = 8:2) and cultured for another 20 min. In panels A and D, the cells were cultured with 1 $\mu$M Go6983 or vehicle for 1 h before 100 nM hPTH(1–34) treatment as indicated. In panel A, 10 $\mu$M Phorbol ester (TPA) was applied to the cells for 30 min (cells could not survive with TPA for 2 h). In B and C, the cells were treated with 100 nM hPTH(1–34) or vehicle for 30 min or 2 h. After the treatment, cells were fixed and the expression of CITED1 was detected with goat polyclonal antibody against CITED1 and Texas red donkey anti-goat IgG. Nuclei were stained with DAPI ($\times$600).

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References


