Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation

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Edited by Peter K. Vogt, The Scripps Research Institute, La Jolla, CA, and approved June 24, 2005 (received for review April 4, 2005)

The FOXO family of forkhead transcription factors plays a key role in a variety of biological processes, including metabolism, cell proliferation, and oxidative stress response. We previously reported that Foxo1, a member of the FOXO family, is regulated through reversible acetylation catalyzed by histone acetyltransferase cAMP-response element-binding protein (CREB)-binding protein (CBP) and NAD-dependent histone deacetylase silent information regulator 2, and that the acetylation at Lys-242, Lys-245, and Lys-262 of Foxo1 attenuates its transcriptional activity. However, the molecular mechanism by which acetylation modulates Foxo1 activity remains unknown. Here, we show that the positive charge of these lysines in Foxo1 contributes to its DNA-binding, and acetylation at these residues by CBP attenuates its ability to bind cognate DNA sequence. Remarkably, we also show that acetylation of Foxo1 increases the levels of its phosphorylation at Ser-253 through the phosphatidylinositol 3-kinase–protein kinase B signaling pathway, and this effect was overridden on the acetylation-deficient Foxo1 mutant. Furthermore, in *in vitro* kinase reactions, the association of wild-type Foxo1 and its target DNA sequence inhibits the protein kinase B-dependent phosphorylation of Foxo1, whereas mutated Foxo1 proteins, which mimic constitutively acetylated states, are efficiently phosphorylated even in the presence of the DNA. These results suggest that acetylation regulates the function of Foxo1 through altering the affinity with the target DNA and the sensitivity for phosphorylation.

FOXO | modification | cAMP-response element-binding protein-binding protein | protein kinase B

The FOXO family of forkhead transcription factors is evolutionally conserved and consists of Foxo1, Foxo3a, Foxo4, and Foxo6 in mammals (1). A recent series of investigations have demonstrated that FOXO factors play key roles in inducing various downstream target genes, including the regulators of metabolism, cell cycle, cell death, and oxidative stress response (1–4). A pivotal regulatory mechanism of FOXO factors is phosphorylation. In response to insulin or several growth factors, FOXO proteins are phosphorylated by protein kinase B (PKB, also known as Akt), a downstream kinase of phosphatidylinositol 3-kinase (PI3K), leading to their translocation from the nucleus to the cytoplasm (5–9). Furthermore, we and others have shown that phosphorylation of Foxo1 and Foxo3a induced by insulin or growth factors target to proteosomal degradation through SCF^{Skp2}-mediated ubiquitination (10–13). Thus, it has been established that phosphorylation and subsequent ubiquitination are fundamental modifications of FOXO factors, resulting in down-regulation of the target gene expression.

Our previous studies revealed that acetylation is a posttranslational modification of FOXO factors (14, 15). cAMP-response element-binding protein (CREB)-binding protein (CBP) triggers the transactivation function of both Foxo1 and Foxo4, whereas following acetylation of these FOXOs by CBP leads to the attenuation of their transcriptional activity (14, 15). Conversely, silent information regulator 2 (Sir2) reverses the acetylation of Foxo1 by its NAD-dependent deacetylase activity and consequently activates transcription mediated by Foxo1 (15). Although this notion has been confirmed in several other studies (16–18) and a proposed action of how acetylation controls the activity of FOXO factors has been reviewed recently (19), its precise regulatory mechanism remains unsolved.

Here, we show that acetylation by CBP at the positively charged basic residues (Lys-242, Lys-245, and Lys-262) in the Foxo1 DNA-binding domain diminishes the ability to interact with a target gene DNA. Moreover, the acetylation promotes the phosphorylation of Foxo1 at Ser-253 through the PI3K–PKB signaling pathway. Whereas DNA-bound Foxo1 resists PKB-dependent phosphorylation *in vitro*, acetylation-mimicked mutants of Foxo1, which have lower affinity with DNA, are efficiently phosphorylated, compared with unacetylated form. These findings suggest a mechanism by which acetylation of Foxo1 destabilizes the Foxo1–DNA complex, and hence PKB readily phosphorylates Foxo1 at the Ser-253 residue.

Materials and Methods

Plasmids and Antibodies. p3×IRS-MLP-luc and pcDNA3-FLAG-Foxo1 were described in refs. 15 and 20. The mutants of Foxo1 acetylation sites, in which lysines (Lys-242, Lys-245, and Lys-262) were replaced by arginine (3KR), alanine (3KA), or glutamine (3KQ) residues, and the mutant of the phosphorylation site, in which Ser-253 was replaced by alanine (SA), were generated by PCR mutagenesis. GST-Foxo1 (amino acids 157–268) was made by PCR-based subcloning into pGEX-5X (Amersham Pharmacia). The following antibodies were used: anti-FLAG (M2, Sigma), anti-hemagglutinin (HA) (12CA5, Roche, Indianapolis), and anti-phospho-Foxo1 (Ser-256), anti-Akt/PKB, and anti-phospho-Akt/PKB (Ser-473) from Cell Signaling Technology (Beverly, MA). Anti-Foxo1 (C3), which recognizes the C terminus of Foxo1, was described in ref. 21. Anti-acetylated Foxo1, which recognizes Foxo1 acetylated at Lys-242 and Lys-245 raised against an acetylated Foxo1 peptide, was described in ref. 15.

Luciferase Assays. HepG2 cells were cultured in DMEM supplemented with 10% FBS and were transfected with indicated plasmids by using GeneJuice transfection reagent (Novagen). pCMV-β-galactosidase plasmid was included to control for the efficiency of transfection, and empty plasmid was added to ensure equal DNA amounts in each transfection. After transfection, cells were incubated in DMEM supplemented with 10% FBS for 30 h, the medium was replaced with serum-free DMEM containing 0.1% BSA, and incubation was continued for 18 h. The luciferase activity was measured and normalized for β-galactosidase activity in the same sample. Luciferase and

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; CBP, cAMP-response element-binding protein (CREB)-binding protein; NIA, nicotinamide; TSA, trichostain A; G6Pase, glucose-6-phosphatase; IP, immunoprecipitation; sir2, silent information regulator 2; IRS, insulin response sequence; HA, hemagglutinin.

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www.pnas.org/cgi/doi/10.1073/pnas.0502738102

β-galactosidase assays were performed in triplicate, and experiments were repeated at least three times.

Immunoprecipitation (IP) and Western Blotting. HEK293T cells were cultured in DMEM supplemented with 10% FBS and were transfected with indicated plasmids. Forty-eight hours after transfection, the cells were treated with 10 mM nicotinamide (NIA), 1 μM trichostatin A (TSA), and 20 μM LY294002 for 6 h, and then lysed in a lysis buffer (50 mM Hepes·KOH, pH 7.9/150 mM NaCl/0.5% Triton X-100/2 mM EDTA/10 mM NIA/1 μM TSA/20 mM NaF/1 mM Na_3VO_4 and protease inhibitors), and the whole-cell extracts were subjected to IP with anti-FLAG (M2) antibody. The cell extracts or immunoprecipitates were resolved by SDS/PAGE followed by electrotransfer onto poly(vinylidene difluoride) membrane and were probed with indicated first antibodies. Chemiluminescent detection relied on horseradish peroxidase-conjugated secondary antibodies.

EMSA. GST-Foxo1 proteins (wild-type, 3KR, 3KA, and 3KQ mutants) encompassing amino acids 157–268 were expressed in *Escherichia coli* BL-21 strain by using the pGEX vector system and purified. A double-stranded oligonucleotide probe containing insulin response sequences (IRSs) derived from the human glucose-6-phosphatase (G6Pase) promoter (between −194 and −160) (22) was end-labeled with ^{32}P. The labeled probe was incubated with 10, 20, or 50 ng of GST-Foxo1 protein in 20 μl of the reaction mixture [20 mM Tris·HCl, pH 8.0/40 mM KCl/5% glycerol/0.4 mM DTT/0.2 mM EDTA/2 mM $MgCl_2$/1 mg/ml BSA and 20 ng of poly(dI-dC)]. After incubation for 15 min on ice, the reaction mixtures were directly loaded onto a 6% polyacrylamide gel and electrophoresed in 0.5× TBE (1× TBE is 89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.3). For the binding-rate measurement, the reaction mixture at room temperature was loaded onto a running polyacrylamide gel to stop the reaction at indicated time points. The gels were dried and analyzed with a bioimaging analyzer (Typhoon 8600, Amersham Pharmacia).

Avidin–Biotin-Conjugated DNA-Binding Assay. HEK293T cells were transfected with the indicated plasmids and treated with NIA and TSA. The whole-cell extracts were incubated with biotinylated 3×IRS DNA, which contained the three copies of the IRS derived from the insulin-like growth factor binding protein-1 promoter (21) and was immobilized on streptavidin–agarose, in a binding buffer [50 mM Hepes·KOH, pH 7.9/150 mM NaCl/0.5% Triton X-100/2 mM EDTA/20 mM NaF/1 mM Na_3VO_4/10 mM NIA/1 μM TSA/20 μg/ml poly(dI-dC) and protease inhibitors] at 4°C for 30 min, then precipitated. The supernatants were recovered and subjected to IP with anti-FLAG antibody. The beads were washed four times with the binding buffer, and precipitated proteins were analyzed by Western blotting.

Chromatin IP Assay. Chromatin IP assay was performed as described in ref. 21, with some modifications. HepG2 cells treated with 20 μM LY294002 in the presence or absence of deacetylase inhibitors (10 mM NIA and 1 μM TSA) for 6 h were crosslinked with 1% formaldehyde for 15 min at 4°C. Chromatin from crosslinked HepG2 cells was sheared by sonication and incubated overnight with anti-Foxo1 antibody or normal rabbit IgG followed by the addition of protein G-Sepharose saturated with salmon sperm DNA. Precipitated DNAs were analyzed by PCR using specific primers for human G6Pase promoter: 5′-AGAATCATCGTGGATGTAGACTCT-3′ and 5′-GCTTGGTGGTGATTGCTCTGCTATG-3′.

Northern Blot Analysis. H4IIE cells were cultured in MEM supplemented with 5% FBS and treated with insulin (100 nM) for 18 h and/or deacetylase inhibitors (10 mM NIA and 1 μM TSA) for 6 h. Total RNA was isolated by using ISOGEN RNA isolation reagent (Nippon Gene, Tokyo) and were denatured with glyoxal, separated on 1.2% agarose, and transferred to a nylon membrane (NEN). The membranes were hybridized with ^{32}P-labeled probes specific for G6Pase and β-actin and analyzed with a bioimaging analyzer.

***In Vitro* Kinase Assay.** To assess the effect of double-stranded DNA on Foxo1 phosphorylation, 1 μg of GST-Foxo1 (amino acids 157–268) was preincubated with 0, 0.5, or 1 pmol of IRS from the G6Pase promoter, 3×IRS from the insulin-like growth factor binding protein-1 promoter, or 3×IRSmut in kinase reaction buffer (20 mM Tris·HCl, pH 8.0/40 mM KCl/0.2 mM EDTA/0.4 mM DTT/2 mM $MgCl_2$/20 mM β-glycerophosphate/50 μM Na_3VO_4) on ice for 15 min, then added 10 ng of activated Akt/PKB (Upstate Biotechnology, Lake Placid, NY) and 0.5 mM ATP. After incubation at 30°C for 15 min, the reaction products were analyzed by Western blotting.

Results

Acetylation of Foxo1 Attenuates Its Site-Specific DNA-Binding. Because we demonstrated in ref. 15 that CBP-induced acetylation of Foxo1 attenuates its transcriptional activity , we next sought to elucidate a mechanism whereby acetylation modulates Foxo1 function(s). First, to verify the consequences of acetylation on Foxo1 transactivation function, we generated several Foxo1 mutants in which the Lys residues of all three CBP-dependent acetylation sites (Lys-242, Lys-245, and Lys-262) were replaced by arginine (3KR), alanine (3KA), or glutamine (3KQ) residues (Fig. 1*A*). Whereas the lysine-to-arginine (KR) substitution avoids acetylation but keeps positive charges, thus mimicking the nonacetylated form, the lysine-to-alanine (KA) or glutamine (KQ) substitutions mimic the constitutively acetylated form through neutralization of the positive charges (23, 24). HepG2 cells were cotransfected with a luciferase construct containing three copies of IRSs and plasmids encoding Foxo1 WT, 3KR, 3KA, or 3KQ. Consistent with our previous findings (15), 3KR substitution enhanced transcriptional activity by 1.5-fold, compared with that of Foxo1 WT (Fig. 1*B*). Conversely, both 3KA or 3KQ substitutions substantially reduced the activity of Foxo1 (Fig. 1*B*), indicating that CBP-dependent acetylation antagonizes the transactivation potential of Foxo1.

It is generally thought that acetylation of transcription factors alters their activity dependent on the functional domains that are modified (25). In the case of Foxo1, the former two lysines acetylated by CBP are located within the basic region of the forkhead DNA-binding domain, and the latter one is adjacent to the C terminus of the basic region (Fig. 1*A*). Notably, x-ray crystallographic studies on the DNA-binding motif of HNF-3γ (also called Foxa3) reported that the positively charged basic residues in the C terminus of the forkhead DNA-binding domain might interact as a random coil with negatively charged phosphate residues in the minor groove of target DNA and stabilize DNA binding (26). Considering this notion along with our findings described above, it was possible that acetylation of Foxo1 on the basic residues in the forkhead DNA-binding motif influences the properties of its DNA-binding, probably by neutralization of the positive charges of lysine residues. To assess this possibility, we performed an EMSA using Foxo1 acetylation site mutants. GST-Foxo1 proteins (amino acids 157–268), which contain the forkhead DNA-binding domain and its C-terminal flanking residues, were tested for their abilities to bind an oligonucleotide probe derived from the promoter region of human G6Pase, one of the FOXO's target genes. As expected, both wild-type and 3KR Foxo1 proteins bound to the probe in a dose-dependent manner (Fig. 1*C*, lanes 2–7). In contrast, both 3KA and 3KQ substitutions, which mimic the acetylated states of

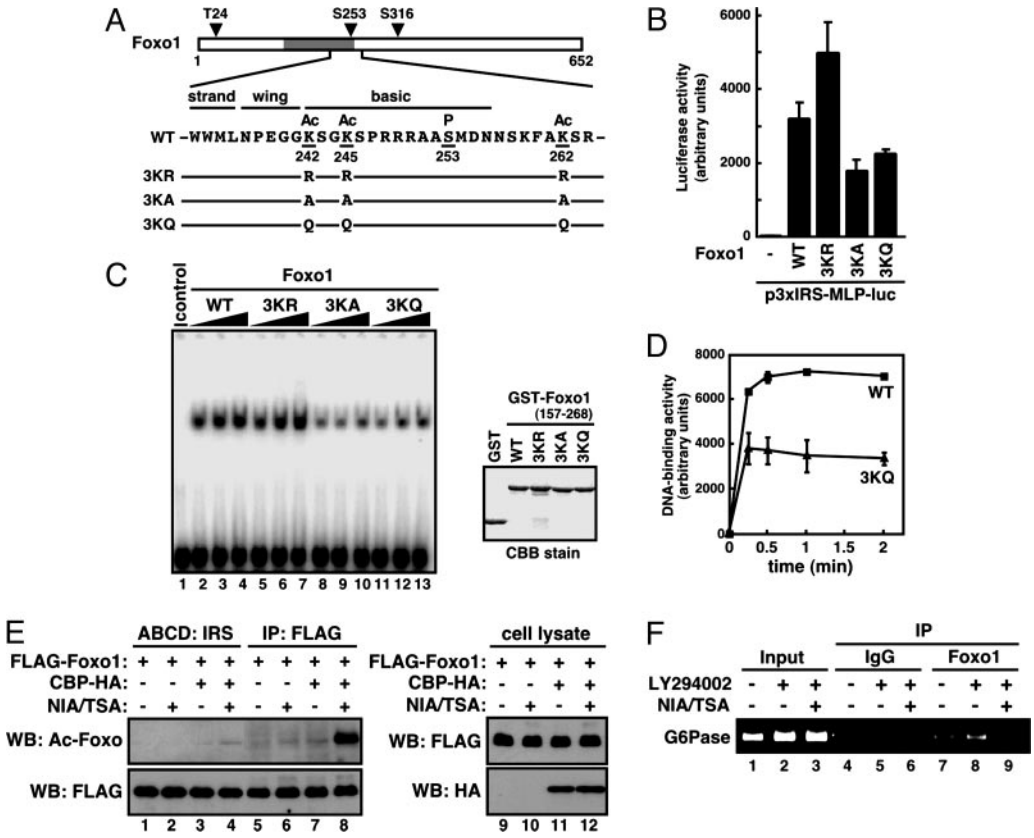

Fig. 1. Acetylation of Foxo1 attenuates DNA-binding and represses its transcriptional activity. (*A*) Schematic representation of Foxo1 point mutants. Lys-242, Lys-245, and Lys-262 are sites acetylated by CBP. Thr-24, Ser-253, and Ser-316 are sites phosphorylated by PKB. The gray box indicates the forkhead DNA-binding domain. (*B*) HepG2 cells were transiently transfected with 50 ng of p3×IRS-MLP-luc and 10 ng of wild-type or mutated Foxo1 expression plasmids, and the luciferase activity was measured. (*C*) EMSA was performed with bacterially expressed wild-type or mutated GST-fusion Foxo1 proteins (amino acids 157–268) and a ^{32}P-labeled double-stranded oligonucleotide probe containing Foxo1-binding sequences from human G6Pase promoter. In the control lane, GST protein was used instead of GST-Foxo1 protein. The amounts of proteins used in the experiment were confirmed by Coomassie brilliant blue stain (*Right*). (*D*) The kinetics of binding of Foxo1 (wild-type or 3KQ mutant) to the probe DNA was measured by EMSA. Bound probe is quantified, and binding activity is shown. (*E*) HEK293T cells transfected with FLAG-Foxo1 and CBP-HA were treated with deacetylase inhibitors NIA and TSA. Whole-cell extracts were incubated with double-stranded 3×IRS immobilized on streptavidin agarose (avidin–biotin-conjugated DNA), and subsequently, the supernatants were recovered and immunoprecipitated with anti-FLAG antibody (IP). After washing the beads, proteins were detected by Western blotting using anti-acetylated-Foxo1 or anti-FLAG antibodies (*Left*). The amounts of FLAG-Foxo1 and CBP-HA in cell extracts were shown by Western blotting with anti-FLAG or anti-HA antibodies (*Right*). (*F*) Chromatin IP assays were performed in HepG2 cells treated with LY294002 and/or deacetylase inhibitors NIA and TSA by using normal rabbit IgG or anti-Foxo1 antibody. Immunoprecipitated (IP) DNA was analyzed by PCR using a primer set specific for G6Pase.

Foxo1, impaired site-specific DNA binding (Fig. 1*C*, lanes 8–13), implying that the neutralization of the basic residues of the forkhead domain by acetylation weakens the affinity between Foxo1 and the target DNA *in vitro*. To further verify the effect of acetylation site mutation on the DNA-binding activity of Foxo1, we measured the kinetics of binding of Foxo1 wild-type or acetylation mimicked 3KQ mutant to the target DNA sequence by a time-course study. As shown in Fig. 1*D*, GST-Foxo1 proteins bound to the probe DNA very rapidly, and binding equilibrium was achieved almost within 15 sec, the earliest time point tested, similar to the results of binding studies previously reported by Zang *et al.* (27). Importantly, the level of Foxo1 3KQ–DNA complex at equilibrium was about half of that of wild type, suggesting that Foxo1 mutation, which mimics acetylated state, decrease the interaction between Foxo1 protein and target DNA.

Next, to determine whether acetylated Foxo1 in cells is suppressed in binding to an IRS-containing DNA, we conducted an avidin–biotin-conjugated DNA-binding followed by IP assay. HEK293T cells were transfected with FLAG-Foxo1 and CBP-HA, and treated with or without a Sir2 inhibitor, NIA, and a class I and II histone deacetylase inhibitor, TSA. Whole-cell extracts were incubated with IRS-containing DNA fragments immobilized on streptavidin beads, and then Foxo1 proteins bound to IRS were precipitated (Fig. 1*E*, lanes 1–4). Thereafter, the unbound Foxo1 proteins in the supernatants were immunoprecipitated by anti-FLAG antibody (Fig. 1*E*, lanes 5–8). Treatment of CBP-transfected cells with deacetylase inhibitors led to a drastic increase in the acetylation of ectopically expressed Foxo1, whereas no acetylated Foxo1 was precipitated with an IRS-containing DNA (Fig. 1*E Upper*, lane 8 vs. lanes 1–4). It should be noted that no differences were observed in the total amount of Foxo1 precipitated with an IRS and an anti-FLAG antibody (Fig. 1*E Lower*, lanes 1–8). These data indicate that nonacetylated Foxo1 interacts with the target DNA sequence more efficiently than does the acetylated form. To further verify the influence of acetylation on DNA-binding activity of Foxo1 *in vivo*, we performed chromatin IP assay in HepG2 cells. As shown in Fig. 1*F*, enrichment of Foxo1 on the G6Pase promoter, especially in the presence of PI3K inhibitor LY294002 (lane 8), was substantially impaired by the treatment with deacetylase inhibitors NIA and TSA (lane 9), suggesting that acetylation of Foxo1 represses its recruitment to the target gene *in vivo*. Thus, acetylation of Foxo1 attenuates its DNA-binding ability, thereby decreasing Foxo1-mediated transcription.

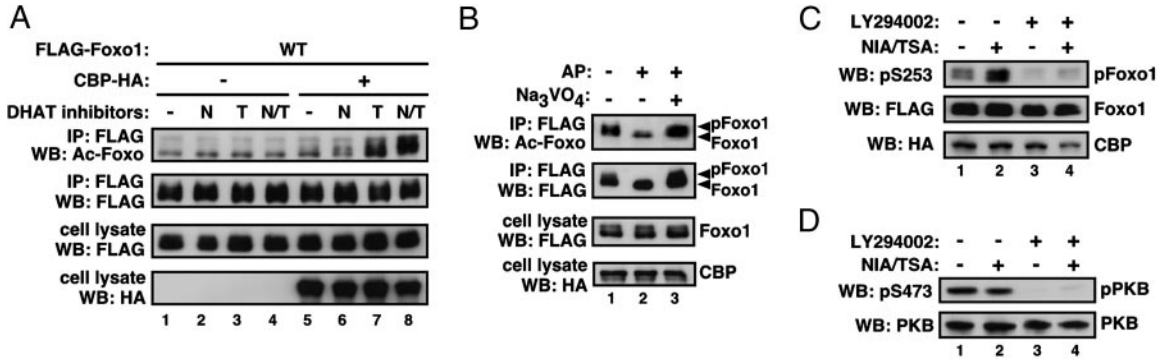

Fig. 2. Deacetylase inhibitors promote phosphorylation of Foxo1. (*A*) HEK293T cells were transfected with FLAG-Foxo1 and CBP-HA and treated with histone deacetylase inhibitors NIA (N) and/or TSA (T). Whole-cell extracts were immunoprecipitated with anti-FLAG antibody, followed by Western blotting (WB). (*B*) HEK293T cells were transfected with FLAG-Foxo1 and CBP-HA and treated with NIA and TSA. FLAG-Foxo1 was immunopurified with anti-FLAG antibody and then incubated with alkaline phosphatase (AP) in the absence or presence of phosphatase inhibitor Na_3VO_4 *in vitro*. Reaction products and cell extracts were analyzed by Western blotting. (*C*) HEK293T cells were transfected with FLAG-Foxo1 and CBP-HA and treated with NIA, TSA, and LY294002. Whole-cell extracts were analyzed by Western blotting. (*D*) HEK293T cells were treated with NIA, TSA, and LY294002. Phosphorylated and total PKB in the whole-cell extracts were analyzed by Western blotting.

Effects of Deacetylase Inhibitors on Foxo1 Phosphorylation. The FOXO family of forkhead transcription factors is negatively regulated by phosphorylation through the PI3K–PKB signaling-pathway (1, 2). Because we have identified CBP-induced acetylation as an additional modification for FOXO factors (14, 15), we next tried to elucidate the relation between acetylation and phosphorylation of Foxo1. To this end, we examined the electrophoretic mobility of Foxo1 proteins in various acetylated states in SDS/PAGE. HEK293T cell extracts expressing FLAG-Foxo1 and CBP-HA were prepared after treatment with deacetylase inhibitors NIA and/or TSA and subjected to Western blot analysis with an anti-acetylated-Foxo1 antibody. As shown in Fig. 2*A*, the coexpression of CBP and the treatment of deacetylase inhibitors prominently increased the levels of Foxo1 acetylation. Strikingly, a large fraction of acetylated Foxo1 migrated as the slow-mobility form (Fig. 2*A*, lane 8). Furthermore, this electrophoretic mobility shift of acetylated Foxo1 was fully reversed by phosphatase treatment of immunoprecipitated Foxo1 *in vitro* (Fig. 2*B*, lane 2), which was counteracted in the presence of phosphatase inhibitor Na_3VO_4 (Fig. 2*B*, lane 3). These results suggest that the observed mobility shift of highly acetylated Foxo1 was a consequence of phosphorylation.

Next, to clarify whether acetylation-induced phosphorylation of Foxo1 is implicated in the PI3K–PKB signaling pathway, we detected the phosphorylation at Ser-253, a major regulatory site for Foxo1 phosphorylation, in cells under the treatment with deacetylase inhibitors NIA and TSA. As shown in Fig. 2C, the phosphorylation level of Foxo1 at Ser-253 was increased when cells were incubated with deacetylase inhibitors (lanes 1 and 2), and moreover, treatment with PI3K-specific inhibitor LY294002 abolished the NIA/TSA-induced phosphorylation (lanes 3 and 4). To exclude the possibility that the deacetylase inhibitors directly stimulated the PI3K–PKB signaling pathway, we measured the phosphorylation level of PKB at Ser-473, which is an indicative of its kinase activity. Although LY294002 indeed inhibited the phosphorylation of PKB, deacetylase inhibitors did not affect it (Fig. 2*D*). Taken together, these findings provide evidence that the acetylation level of Foxo1 mutually correlates with the phosphorylation level.

Effects of Acetylation Site Mutations on Foxo1 Phosphorylation. To further confirm the contribution of the acetylation of Foxo1 at the three identified lysines (Lys-242, Lys-245, and Lys-262) to phosphorylation, we tested whether the phosphorylation level at Ser-253 is altered on acetylation-deficient Foxo1 3KR mutant after the deacetylase inhibitor treatment. Significantly, the treatment with deacetylase inhibitors had no effect on the phosphorylation level of Foxo1 3KR (Fig. 3*A*), suggesting that the acetylation at these lysine residues is important for promotion of Foxo1 phosphorylation in response to deacetylase inhibitors. Supporting this idea, Foxo1 3KA and 3KQ mutants, both of which mimic the constitutively acetylated form, were more highly phosphorylated, compared with WT or 3KR Foxo1 (Fig. 3*B*). However, the acetylation of Foxo1 appeared not to be prerequisite for phosphorylation, because under normal conditions, Foxo1 3KR also was phosphorylated in a similar extent to Foxo1 WT (Fig. 3 *A* and *B*). Taking these results together, we conclude

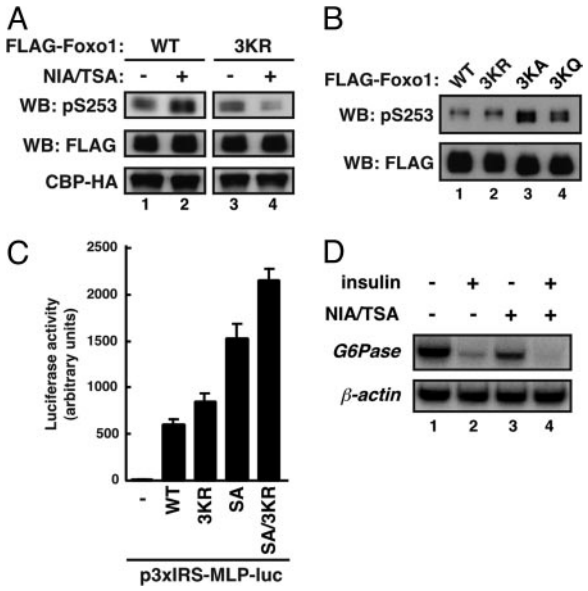

Fig. 3. The mutations at acetylation sites affect the phosphorylation level of Foxo1. (*A*) HEK293T cells were transfected with FLAG-Foxo1 (wild-type or 3KR mutant) and CBP-HA, and treated with NIA and TSA. (*B*) HEK293T cells were transfected with FLAG-Foxo1 (wild-type or indicated mutants). Whole-cell extracts were analyzed by Western blotting. (*C*) HepG2 cells were transiently transfected with 50 ng of p3×IRS-MLP-luc together with 5 ng of the indicated Foxo1 expression plasmids, and the luciferase activity was measured. (*D*) H4IIE cells were treated with insulin and/or deacetylase inhibitors NIA and TSA. Total RNA was hybridized with ^{32}P-labeled probes specific for G6Pase and β-actin genes.

that the acetylated form of Foxo1 raises the sensitivity to its phosphorylation via the PI3K–PKB signaling pathway.

Because each modification of Foxo1, phosphorylation and acetylation, regulates its transcriptional activity, we examined the functional relevance of these modifications to the Foxo1-mediated transcription. We introduced a point mutation of Ser-253 to alanine into the Foxo1 3KR mutant (SA/3KR), which is deficient in both acetylation and phosphorylation. HepG2 cells were cotransfected with an IRS-containing luciferase construct and plasmids encoding Foxo1 WT, 3KR, SA, or SA/3KR. Compared with WT Foxo1, 3KR and SA mutants activated transcription by 1.4- and 2.5-fold, respectively, and SA/3KR mutant prominently potentiated the promoter activity by 3.6-fold (Fig. 3*C*). These results suggest that acetylation and phosphorylation cooperatively repress the transcriptional activity of Foxo1. Furthermore, we assessed the effects of insulin and deacetylase inhibitors on the expression of endogenous Foxo1 target gene, G6Pase. H4IIE cells were treated with insulin and deacetylase inhibitors and total RNA was isolated and then subjected to Northern blot analysis. As expected, the treatment with insulin remarkably suppressed the expression of G6Pase (Fig. 3*D*, lanes 1 and 2), whereas its expression was moderately reduced in the presence of deacetylase inhibitors (lane 3). Significantly, the treatment with both insulin and deacetylase inhibitors completely reduced the G6Pase expression (Fig. 3*D*, lane 4), implying that the phosphorylation and acetylation of endogenous Foxo1 down-regulate the expression of the endogenous target gene.

Effects of Acetylation Site Mutations on Phosphorylation of DNA-Bound Foxo1. Given our initial finding that acetylation of Foxo1 attenuates its DNA-binding ability (Fig. 1), we hypothesized the mechanism by which acetylation of Foxo1 increases the sensitivity of PKB-dependent phosphorylation, namely, Foxo1 acetylation mediated by CBP might weaken the Foxo1–DNA association, thereby facilitating the access of PKB to the Ser-253 residue of Foxo1. To address this hypothesis, we performed *in vitro* kinase assays to estimate the efficiency of PKB-mediated phosphorylation in the presence of DNA fragments. GST-Foxo1 (amino acids 157–268) was phosphorylated at Ser-253 by purified PKB in an ATP-dependent manner *in vitro* (Fig. 4*A*). As shown in Fig. 4*B*, preincubation of Foxo1 protein with the DNA fragments containing IRS derived from G6Pase or insulin-like growth factor binding protein-1 promoter exhibited a dose-dependent inhibition in PKB-mediated phosphorylation (lanes 2–5), whereas preincubation with the mutated IRS fragment had no effect on the phosphorylation of Foxo1 (lanes 6 and 7). This result suggests that Foxo1 bound to its cognate DNA sequence is protected from the phosphorylation by PKB. In addition, to investigate whether CBP-mediated acetylation antagonizes the inhibitory effects of IRS on Foxo1 phosphorylation, we conducted the kinase assays by using various Foxo1 acetylation site mutants. Wild-type and mutated Foxo1 proteins were almost equally phosphorylated by PKB *in vitro* (Fig. 4*C*). Remarkably, although preincubation of Foxo1 proteins with the IRS fragments substantially inhibited phosphorylation of Foxo1 WT and 3KR proteins (Fig. 4*D*, lanes 1–6), no differences were observed in the phosphorylation levels of Foxo1 3KA and 3KQ proteins, even in the presence of the IRS fragments (Fig. 4*D*, lanes 7–12). Considering the lower affinity of the acetylation-mimicked mutants (3KA and 3KQ) with the IRS-containing DNA (Fig. 1 *C* and *D*), it is possible that acetylation of Foxo1 augments its phosphorylation by attenuating the affinity between Foxo1 and DNA, consequently increasing the accessibility of PKB to Foxo1.

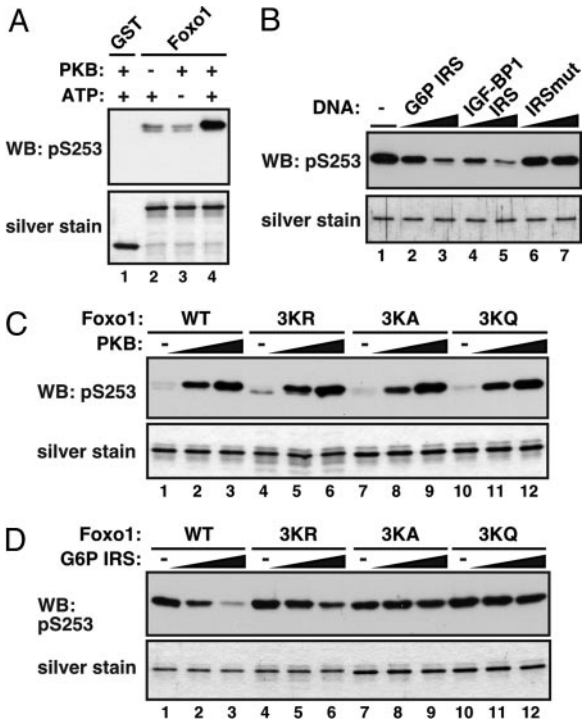

Fig. 4. The association of Foxo1 and its cognate DNA sequence inhibits the PKB-dependent phosphorylation of Foxo1 *in vitro*. (*A*) *In vitro* kinase assays were performed with 1 μg of GST-Foxo1 (amino acids 157–268), 10 ng of PKB, and 0.5 mM ATP. (*B*) One microgram of wild-type GST-Foxo1 (amino acids 157–268) was preincubated with the indicated double-stranded oligonucleotides (0.5 or 1 pmol) and phosphorylated by 10 ng of PKB and 0.5 mM ATP *in vitro*. (*C*) Wild-type or mutated GST-Foxo1 (1 μg) was phosphorylated by PKB (0, 2, or 10 ng). (*D*) Wild-type or mutated GST-Foxo1 (1 μg) was preincubated with double-stranded oligonucleotide (0, 0.5, or 1 pmol) and phosphorylated by 10 ng of PKB. All reaction products were analyzed by Western blotting using anti-phospho-Foxo1 antibody or silver stain.

Discussion

Our present study provides two findings about the consequences of Foxo1 acetylation, the attenuation of DNA-binding ability and the enhancement of the level of Foxo1 phosphorylation. We further explored the relevance of DNA binding to Foxo1 phosphorylation and revealed that the interaction between Foxo1 and IRS fragments prevents its PKB-dependent phosphorylation, whereas acetylation-mimicked mutations of Foxo1 antagonize the inhibitory effect of IRS, and those mutants are more sensitive to phosphorylation. In a recent review article, van der Heide and Smidt (19) speculated on the influence of acetylation on DNA-binding ability and phosphorylation of FOXO factors, and in this study, we indeed presented evidence of the mechanism by which acetylation inhibits the activity of Foxo1.

Based on the results shown above, a possible model can be drawn (Fig. 5). In the nucleus, Foxo1 recognizes and binds to the IRSs on a target gene promoter. CBP should be recruited to IRS-bound Foxo1 and stimulate the transcription by acety-

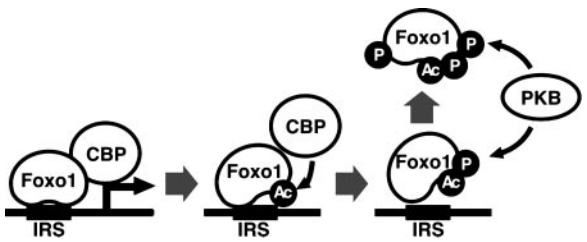

Fig. 5. A model for Foxo1 regulation through acetylation and phosphorylation.

lating nucleosomal histones, whereas subsequent CBP-induced acetylation on the basic region of the forkhead DNA-binding domain impairs the interaction between Foxo1 and IRS. Accordingly, activated PKB could efficiently phosphorylate acetylated-Foxo1 at Ser-253, which would be a gatekeeper of phosphorylation (28, 29), leading to following phosphorylation of Foxo1 at other residues and, in turn, nuclear exclusion to the cytoplasm.

Intriguingly, Zhang *et al.* (27) have reported that the basic residues in the forkhead domain are important for DNA binding, and the introduction of a negative charge by phosphorylation at Ser-256 of human FOXO1 diminishes its DNA-binding ability. They also argued that FOXO1 DNA-binding is rapid and reversible, and that this unstable state would provide the opportunity for PKB to phosphorylate at Ser-256, thus further reducing the binding of FOXO1 to DNA, and hence additional phosphorylation at other sites occurs. In view of our present results, it is possible that acetylation at the basic region in the forkhead DNA-binding domain is an initial step to facilitate the dissociation of Foxo1 from DNA, and consequently, PKB readily phosphorylates Foxo1 at Ser-253.

More recently, Frescas *et al.* (30) have reported the effect of SIRT1 (a human Sir2 ortholog)-dependent deacetylation on Foxo1 subcellular localization by a series of examinations using living cell imaging. They have shown that, in cells treated with H_2O_2 or resveratrol, an activator of SIRT1, Foxo1 remains within the nucleus, overcoming the effect of insulin, probably because insulin-induced phosphorylation of Foxo1 is abolished and Foxo1 remains to be bound to the nuclear compartment. Conversely, treatment with NIA, an inhibitor of SIRT1, increases the acetylation of Foxo1 and prevents H_2O_2- or resveratrol-induced nuclear localization of Foxo1. Given our previous study describing that Sir2 deacetylates Foxo1 (15), a plausible explanation could be drawn that deacetylation of Foxo1 at Lys-242, Lys-245, and Lys-262 by activated Sir2 would reinforce the interaction with IRS and disturb the PKB-induced phosphorylation at Ser-253, thereby promoting the nuclear accumulation of Foxo1. On the other hand, when Sir2 is inactive, Foxo1 highly acetylated by CBP would dissociate from DNA, which increases the opportunity for PKB to phosphorylate Foxo1 at Ser-253, resulting in its further phosphorylation and rapid translocation in the cells.

Our studies demonstrate that acetylation of Foxo1 leads to the decrease in its DNA-binding activity. Acetylated Foxo1 becomes more sensitive to PKB-dependent phosphorylation, suggesting that acetylation and phosphorylation cooperatively regulate the function of Foxo1. This concept based on the dual posttranslational modifications provides additional dimension to the regulatory mechanism of Foxo1, whose complexity converges on Foxo1 to finely control its transactivation function.

We thank the Fukamizu laboratory members for their helpful discussion. This work was supported by the 21st Century Center of Excellence Program and Grants-in-Aid for Scientific Research (Category A), Scientific Research on Priority Areas, and Young Scientists (Category B) from the Ministry of Education, Science, Sports, and Technology of Japan.

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