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Regulation of Intracellular Localization and Transcriptional Activity of FOXO4 by Protein Kinase B through Phosphorylation at the Motif Sites Conserved among the FOXO Family

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FOXO4 transcription factor, also referred to AFX, contains three putative phosphorylation motif sites for protein kinase B (PKB), Thr32, Ser197, and Ser262, and it is proposed that phosphorylated FOXO4 stays in the cytosol and is imported to the nucleus through dephosphorylation to induce target gene expression. These three sites were revealed to be phosphorylated by PKB *in vitro* on phosphopeptide analysis, and in cultured cells on immunoblotting with phosphorylation-site specific antibodies. The mutants with either Thr32 or Ser197 replaced by Ala were found mostly in the nuclear but not the cytosol fraction, and treatment with platelet-derived growth factor did not change their distributions in the cells. FOXO4 proteins mutated at these two sites showed 3- to 5-fold higher transcriptional activity than that of the wild type. In contrast, the replacement of Ser262 did not alter the localization or transcriptional activity. These results indicate that phosphorylation at Thr32 and Ser197 is indispensable, whereas that at Ser262 is not critical, for regulation of the nuclear localization and transcriptional activity of FOXO4. These properties are similar to those of FOXO1 and FOXO3, and thus FOXO transcription factors seem to be regulated through a common mechanism by PKB in the growth factor signaling pathway.

Key words: FOXO4, nuclear localization, PKB, phosphorylation, transcription factor.

Abbreviations: DBE, daf-16 family protein–binding element; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GST, glutathione *S*-transferase: MBP, maltose-binding protein; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI, phosphatidylinositol; PKB, protein kinase B.

Protein kinase B (PKB, also named Akt) comprises a family of Ser/Thr protein kinases having pleckstrin homology (PH) and catalytic domains in the amino- and carboxyl-terminal halves, respectively, that mediates the signal of growth factors (*1*, *2*). There are three subtypes of PKB in mammals, PKBα (Akt1), PKBβ (Akt2), and PKBγ (Akt3). Mostly through studies on PKBα, it has been revealed PKB family members associate with phosphatidylinositol (PI) 3-kinase products in the plasma membrane through the PH domain and are phosphorylated in the activation loop by an upstream kinase, PDK1, upon cell stimulation. The PKB subtypes thus activated contribute to the control of cellular processes such as metabolism, cell proliferation, differentiation, and apoptosis by phosphorylating various substrate proteins.

The Forkhead domain was first identified as a DNA-binding region of approximately 110 amino acid residues in the *Drosophila* fork head gene and rat hepatocyte nuclear factor 3 (HNF-3) (*3*), and more than one hundred transcription factors containing this domain have been isolated since then from species from yeast to mammals (*4*). The FoxO (forkhead box, class O) subfamily of this gene family in mammals such as FOXO1, FOXO3, and FOXO4, also referred to as FKHR, FKHR-L1, and AFX, respectively, have been revealed to be downstream targets in the signaling pathway of PI 3-kinase and PKB (*5*–*7*). Initially, genetic studies on *Caenorhabditis elegans* indicated that *Daf-16*, the ortholog of the mammalian FOXO, is located downstream of *Daf-2*, *AGE-1*, and *Ce-Akt*, which are counterparts of the mammalian insulin receptor, catalytic subunit of PI 3-kinase, and PKB, respectively, and that *Daf-16* is responsible for dauer formation through the transcription of its target genes when the *Daf-2* signaling pathway is inactivated (*8*, *9*). Furthermore, *Daf-16* was proposed to be a direct target of *Ce-Akt* in *C. elegans*, because it contains three PKB phosphorylation consensus motif sites, Arg-X-Arg-X-X-Ser/Thr (*10*). These motif sites, one Thr and two Ser residues, are conserved among FOXO1, FOXO3, and FOXO4 in mammals (*9*), and are named T1, S1, and S2 (*5*).

These FOXO transcription factors have both nuclear localization and export signals, and are shuttled between the cytosol and nucleus. It has been revealed that these FOXO members are direct targets of PKB and that their intracellular localization is regulated through phosphorylation by PKB (*11*–*17*). It is proposed that they stay in the cytosol in association with 14-3-3 proteins through the PKB phosphorylation reaction at these sites and

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dephosphorylation causes nuclear import to induce the expression of a series of target proteins that regulate metabolism, the cell cycle, and apoptosis. The phosphorylation sites mediated by PKB vary, however, among FOXO1, FOXO3, and FOXO4. Concerning FOXO1, Biggs *et al*. (*11*) reported that the T1 and S1 sites are phosphorylated by PKB, and that mutants in which these residues are replaced are resistant to both PKB-mediated phosphorylation *in vitro* and PI 3-kinase–stimulated nuclear export. Rena *et al*. (*12*) found that the three motif sites of FOXO1 are recognized by PKB *in vitro* and are phosphorylated *in vivo* using phosphorylation site–specific antibodies. In FOXO3, these three motif sites were phosphorylated *in vivo*, and PKB recognized the T1 and S1 sites as in the case of FOXO1, but PKB did not phosphorylate the S2 site (*13*). Therefore, the T1 and S1 sites are commonly phosphorylated by PKB in FOXO1 and FOXO3. In the case of FOXO4, in contrast, Kops *et al*. (*14*) showed that human FOXO4 is phosphorylated at Ser 193 (S1 site) and Ser262 (S2 site), but Thr28 (T1 site) is not modified either *in vitro* or *in vivo*. Moreover, this group did not detect any phosphothreonine in the wild type FOXO4 protein after the phosphorylation reaction with PKB (*14*). We proposed, however, that the three sites are equally important for regulation of FOXO4 based on observation of the intracellular localization of the green fluorescent proteins-fused with the single mutants of FOXO4 with each residue replaced by Ala (*15*). Therefore, different results have been obtained regarding the role of the T1 site in FOXO4. Based on the initial study on this transcription factor (*14*), it is regarded that the role of phosphorylation in FOXO4 is apparently distinct from that in FOXO1 and FOXO3.

Recent analysis of human (*18*) as well as mouse (*19*) clones revealed that authentic human FOXO4 has four amino acid residues at the amino-terminal end in addition to the clone employed in the previous studies (*14*, *15*). Therefore, we analyzed the role of phosphorylation of FOXO4 using the authentic human FOXO4 clone in this study.

MATERIALS AND METHODS

Vector Construction—cDNA of FOXO4 was isolated from human heart, brain, and testis cDNA libraries (CLONTECH) by PCR according to the registered sequence (accession number X93996) (*20*). The sequences of the obtained clones contained four extra amino acid residues at the amino-terminal end in addition to the deduced sequence of the previous clone, and were consistent with AFXα (accession number Y11284) (*18*) with an open reading frame of 505 amino acid residues. Thus, the FOXO4 employed in this study has Thr32, Ser197, and Ser262 as putative PKB phosphorylation motif sites. The cDNA fragment corresponding to amino acid residues 2–505 of FOXO4 isolated was introduced into the pTB701-FLAG and pMAL-c2 vectors. The resulting expression plasmids having the FLAG-epitope tag and maltose-binding protein (MBP) sequences at the amino-terminus were designated as FLAG-FOXO4 and MBP-FOXO4, respectively. Three putative phosphorylation sites were each replaced by Ala through site-directed mutagenesis, and the resulting point mutants were referred to as T32A, S197A, and S262A, respectively. Mutant molecules having Ala at two out of the three sites and a mutant with all three residues replaced were generated through sequential site-directed mutagenesis, and were designated as T32A/S197A, T32A/S262A, S197A/S262A, and the triple mutant (TM), respectively. The cDNA fragments corresponding to the wild type FLAG-FOXO4 and the mutants were amplified with PCR, and then cloned into pMXs-puro obtained from Dr. T. Kitamura (University of Tokyo). The resultant retroviral constructs were designated as pMXs-FLAG-FOXO4, pMXs-FLAG-FOXO4 T32A, pMXs-FLAG-FOXO4 S197A, pMXs-FLAG-FOXO4 S262A, pMXs-FLAG-FOXO4 T32A/S197A, pMXs-FLAG-FOXO4 T32A/S262A, pMXs-FLAG-FOXO4 S197A/S262A, and the pMXs-FLAG-FOXO4 triple mutant, respectively. The DNA sequences of these constructs were confirmed by the dideoxynucleotide chain–termination method using a DNA Sequencing System model 3100 Avant (Applied Biosystems). Recombinant retroviruses were produced by transfection of the retroviral constructs into plat-E virus packaging cells (*21*), and the medium containing each recombinant retrovirus was harvested and stored at –80°C until use. The baculovirus vector of the glutathione *S*-transferase (GST)-fusion protein of rat PKBα (GST-PKBα) was prepared as described previously (*22*). The p6xDBE-luc plasmid, containing the daf-16 family protein–binding element 5′-upstream of the firefly luciferase gene, was provided by Dr. T. Furuyama (National Institute for Longevity Sciences) (*19*). pRL-SV40 having the *Renilla* luciferase gene under the control of the SV40 promoter was purchased from Promega.

Antibodies—The monoclonal anti-FLAG and anti–α-tubulin antibodies were obtained from Sigma. The antibodies against FOXO4 and lamin B were purchased from Santa Cruz. The anti-PKBα antibody and the antibody selectively recognizing phosphorylated Ser197 of FOXO4 were obtained from Pharmingen and Cell Signaling, respectively. The polyclonal antibodies directed against FOXO4 phosphorylated at Thr32 and Ser262 were raised essentially as described (*23*) by immunizing rabbits with phosphopeptides CRPRSCpTWPLPR and CFRPRSSpSNASSVS coupled to keyhole limpet hemocyanin, there being an additional cysteine residue at the amino-terminal ends of the amino acid sequences of 27–37 and 256–268 of human FOXO4, where pT and pS correspond to phosphothreonine and phosphoserine, respectively. The antibodies were purified by successive column chromatographies on resins coupled with the phosphopeptides and non-phosphopeptides. Where indicated, the antibodies generated were adsorbed with each antigen phosphopeptide. These three phosphorylation site–specific antibodies were designated as anti-pSer197, anti-pThr32, anti-pSer262, respectively. The alkaline phosphatase–conjugated anti-mouse and anti-rabbit antibodies, and the horseradish peroxidase–conjugated anti-mouse antibody were purchased from Chemicon.

Cell Culture and Recombinant Protein Production—COS-7 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque) supplemented with 10% fetal calf serum (FCS) and 10% calf serum, respectively, at 37°C in a 5% CO_2 incubator. COS-7 cells were transfected with each expression plasmid by electroporation using Gene Pulsar (Bio-Rad), and then cultured in DMEM supplemented with 10% FCS for 36 h. NIH 3T3

cells were infected with recombinant retroviruses, or transfected using Fugene 6 transfection reagent (Roche) for the reporter assay according to the manufacturer's protocol. For infection, exponentially growing NIH 3T3 cells were exposed to a 1:5 dilution of recombinant retrovirus and fresh medium in two consecutive rounds with 8 μg/ml of polybrene. After incubation for 24 h, the cells were starved for 20 h and then treated with 20 ng/ml platelet-derived growth factor (PDGF) for 40 min. The cells harvested from two 10-cm dishes were subjected to subcellular fractionation using NE-PER (Pierce), which yielded approximately 150 μg and 50 μg of proteins for the cytosolic and nuclear fractions, respectively, as measured with BCA protein assay kit (Pierce). Insect Sf-9 cells were cultured and infected with the baculovirus vector GST-PKBα, and the recombinant protein was purified as described previously (*22*). The wild type MBP-FOXO4 and its mutants were expressed in *Escherichia coli* strain BL21 codon plus RIL (Stratagene). The recombinant proteins were purified by sequential column chromatographies on amylose resin (New England Biolabs) and HiTrap Q Sepharose (Amersham).

In Vitro Phosphorylation and Phosphopeptide Mapping—The MBP-FOXO4 proteins (1 μg each) were incubated with and without GST-PKBα (100 ng) in 20 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 20 μM ATP, and 15–50 kBq of [γ-^{32}P]ATP at 30°C for 30 min. After boiling in SDS-sample buffer, MBP-FOXO4 was separated by SDS-PAGE. The ^{32}P-labeled proteins were recovered from the gel, and then tryptic phosphopeptide mapping was carried out using *N*-tosyl-L-phenylalanine chloromethyl ketone-trypsin (Nacalai Tesque) as described (*24*). The first electrophoretic dimension was performed at 1 kV for 25–35 min at pH 8.9. The second dimension was performed by ascending chromatography in phosphochromo buffer until the solvent front was 2.5 cm from the top of the chromatography plate. The radioactivity incorporated into the MBP-FOXO4 proteins and tryptic fragments was analyzed using Bio-imaging analyzer BAS-2500 (Fuji). When the samples were subjected to immunoblot analysis, the reaction was carried out in the absence of [γ-^{32}P]ATP.

Immunoprecipitation and Immunoblot Analysis—Immunoprecipitation was carried out at 0–4°C essentially as described (*25*). Briefly, COS-7 cells were washed with ice-cold phosphate-buffered saline and then lysed in 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, and 50 μg/ml phenylmethylsulfonyl fluoride. The lysate was centrifuged at 18,000 × *g* for 10 min, and the resulting supernatant (500–600 μg protein) was incubated for 1 h with the anti-FLAG monoclonal antibody. Then, protein A–Sepharose (Amersham) was added to the mixture, followed by incubation for 30 min. The immunoprecipitate was collected by centrifugation, and washed with 20 mM Tris-HCl, pH 7.5, containing 1% Triton X-100 and 150 mM NaCl. The immunoprecipitates, MBP-FOXO4 phosphorylated *in vitro*, and cytosolic and nuclear fractions were boiled in SDS-sample buffer, and the proteins were separated by SDS-PAGE and then transferred to an Immobilon P membrane (Millipore). After incubation with each primary antibody, the alkaline phosphatase–conjugated anti-mouse and anti-rabbit antibodies were employed as the secondary antibodies. The color reaction was carried out using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium as substrates. Where indicated, the horseradish peroxidase-conjugated anti-mouse antibody was employed as the secondary antibody and the chemiluminescence reaction was carried out using ECL plus chemiluminescence detection kit (Amersham).

Reporter Assay—NIH 3T3 cells growing on 24-well plates (1×10^4 cells/well) were transfected with reporter plasmids, p6xDBE-luc and pRL-SV40, and the wild type and mutants of FLAG-FOXO4. After incubation for 24 h, the relative luciferase activity, *i.e.*, firefly enzyme activity divided by that of the *Renilla* enzyme, was determined using Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

RESULTS

In Vitro Phosphorylation Sites for PKB—FOXO4 contains three putative phosphorylation motif sites for PKB, *i.e.*, Thr32, Ser197, and Ser262 (Fig. 1A). To examine the

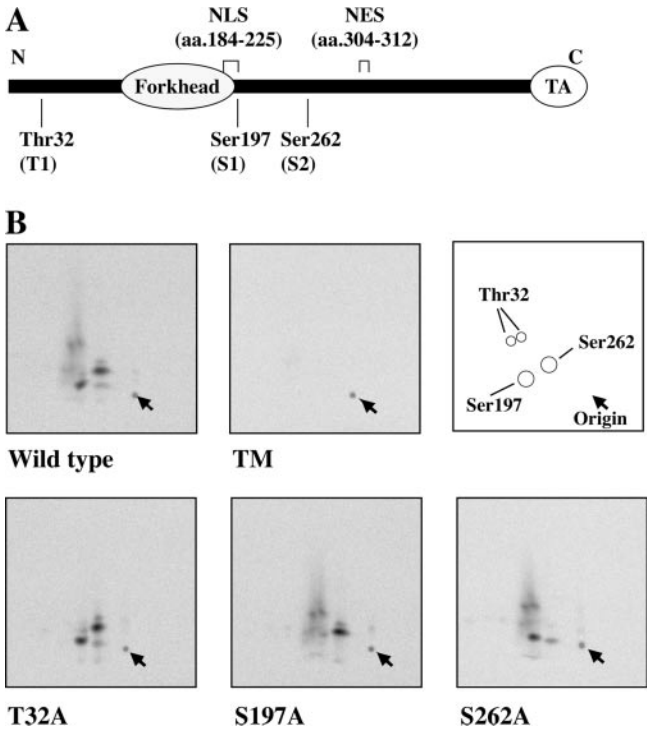

Fig. 1. **Phosphorylation of FOXO4 by PKB *in vitro*.** (A) The structure of FOXO4 is presented schematically. FOXO4 contains DNA-binding (Forkhead) and transactivation (TA) domains in the amino- and carboxyl-terminal regions, respectively. The positions of three putative PKB-motif sites (T1, S1, and S2) are shown with the amino acid residues, and the nuclear localizing signal (NLS) and nuclear export signal (NES) are indicated by amino acid residue numbers. (B) MBP-FOXO4 proteins expressed in bacteria were incubated with purified active GST-PKBα *in vitro*, and then subjected to two-dimensional phospho-peptide mapping. The origin is indicated by arrows, and the spots of phospho-peptides containing the PKB phosphorylation motif sites are indicated in the upper right panel. The data are representative of three independent experiments.

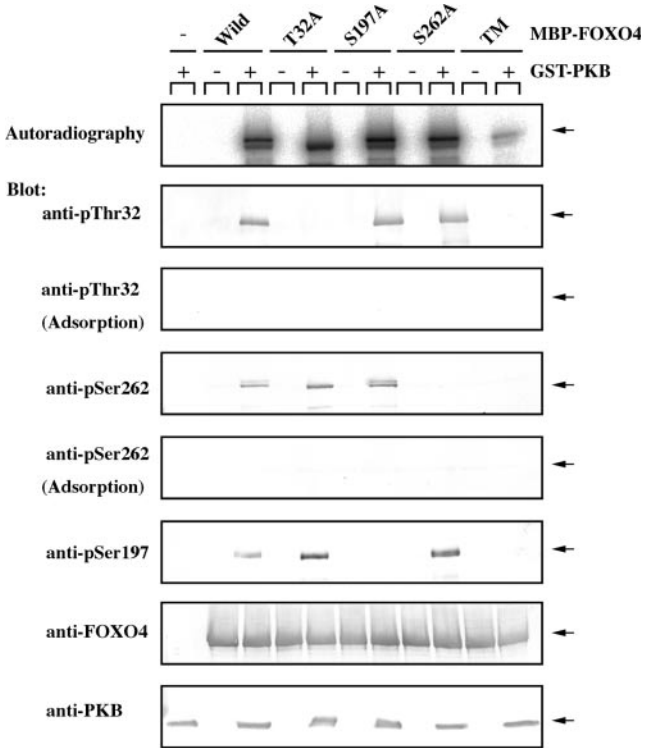

Fig. 2. **Phosphorylation site–specific antibodies.** MBP-FOXO4 proteins expressed in bacteria were phosphorylated by purified active GST-PKBα *in vitro*. Phosphorylation of MBP-FOXO4 is shown by autoradiography in the top panel, and immunoblot analysis with each antibody is shown in lower panels. Where indicated as adsorption, the antibodies were pre-incubated with each phosphopeptide used as the antigen. The positions of MBP-FOXO4 and PKB are indicated by arrows. The data are representative of three independent experiments.

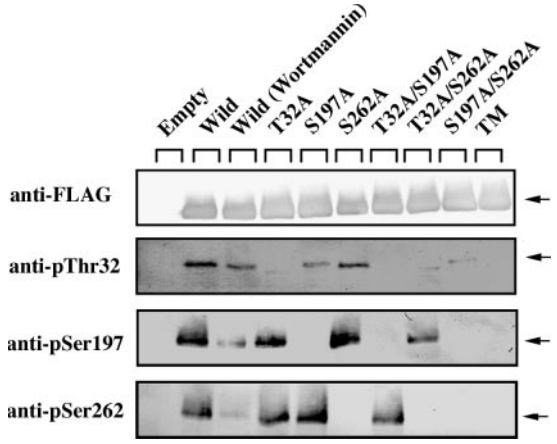

Fig. 3. **Phosphorylation of FOXO4 in COS-7 cells.** FLAG-FOXO4 proteins were expressed in COS-7 cells, immunoprecipitated with the anti-FLAG antibody, and then subjected to immunoblot analysis with the anti-FLAG antibody and phosphorylation site–specific antibodies. Where indicated, cells expressing the wild type FLAG-FOXO4 were treated with 200 nM wortmannin for 1 h. The positions of FLAG-FOXO4 are indicated by arrows. The data are representative of three independent experiments.

phosphorylation by PKB at these residues, the recombinant MBP-FOXO4 proteins were incubated with active PKB in the presence of [γ-^{32}P]ATP. The wild type FOXO4 was significantly phosphorylated, and slight phosphorylation was still observed for the triple mutant with these three residues replaced by Ala (Fig. 2, top panel). Namely, FOXO4 is phosphorylated by PKB at these three mutation sites (*14*, *15*), and has some minor phosphorylation site(s) for PKB in addition to these motif sites. Single mutant molecules, in which one phosphorylation residue was replaced by Ala, were phosphorylated almost as efficiently as the wild type protein. Then, the phosphorylation sites were analyzed by tryptic phosphopeptide mapping (Fig. 1B). Some major phosphopeptides were detected in the wild type FOXO4 that were not found in the triple mutant. One of the different major phosphopeptides was not detected in S197A and S262A, respectively, and in addition, two adjacent spots, presumably containing the same phosphorylated residue, were absent for T32A. The results indicate that PKB predominantly phosphorylates at Thr32, Ser197, and Ser262 of FOXO4 *in vitro*, as summarized in the upper right panel of the figure.

Phosphorylation In Vivo—Antibodies against phosphopeptides corresponding to the region including phosphorylated Thr32 and Ser262, respectively, were generated to analyze the phosphorylation at these motif sites. Immunoblot analysis revealed that the anti-pThr32 antibody recognized the wild type MBP-FOXO4, which was incubated with active PKB *in vitro*, but did not react with the unphosphorylated wild type protein (Fig. 2). This antibody detected S197A and S262A incubated with active PKB, but did not react with either T32A or the triple mutant. The anti-pThr32 antibody pre-incubated with the antigen peptide did not react with the phosphorylated FOXO4 proteins. These data indicate that the anti-pThr32 antibody specifically recognizes FOXO4 phosphorylated at Thr32. Similar results were obtained for the anti-pSer262 antibody. These phosphorylation site–specific antibodies were employed to analyze the phosphorylation of FOXO4 in the cells together with the commercial antibody that recognizes FOXO phosphorylated at Ser197 (Fig. 2). The FLAG-epitope tagged wild type FOXO4, which was immunoprecipitated from COS-7 cells cultured with serum, was recognized by these three phosphorylation site–specific antibodies (Fig. 3). The treatment of the cells with wortmannin, a potent inhibitor of PI 3-kinase, greatly attenuated the immunoreaction of these antibodies. Furthermore, the anti-pThr32 antibody recognized S197A, S262A and S197A/S262A in addition to the wild type, but did not react with T32A, T32A/ S197A, T32A/ S262A, or the triple mutant expressed in the cells. Similar results were obtained with the anti-pSer197 and anti-pSer262 antibodies. These results indicate that these three phosphorylation motif sites are phosphorylated in a manner dependent on PI 3-kinase, presumably by PKB. It is suggested that, in FOXO1, the S1 site is phosphorylated initially, the T1 and S2 sites being subsequently phosphorylated (*26*). The data obtained suggest, however, that these three motif sites are phosphorylated independently in FOXO4 *in vivo*. Therefore, these mutant molecules were employed to study the role of phosphorylation of FOXO4 at each of the three motif sites in the following analysis.

Intracellular Localization—PKB is activated by growth factor stimulation through the PI 3-kinase dependent pathway, and it has been reported that the intracellular localization and transcriptional activity of FOXO4, as well as those of some other family members such as FOXO1 and FOXO3, are regulated by phosphorylation at PKB motif sites (*11–17*). Thus, the role of phosphorylation in the localization of the transcription factor was studied employing NIH 3T3 cells expressing the FLAG-FOXO4 proteins by using recombinant retroviruses (Fig. 4). In NIH 3T3 cells, PKB is activated through PDGF stimulation (*25*, *27*). The cells were serum-starved, stimulated with PDGF, which activates the PI 3-kinase/PKB pathway, and then separated into cytosol and nuclear fractions to compare the distribution of each FLAG-FOXO4 protein. The subcellular fractionation was confirmed by observing the localization of α-tubulin and lamin B as marker proteins of the cytosol and nucleus, respectively. In resting cells, the wild type FOXO4 was detected mainly in the cytosol fraction, with a small amount of the protein in the nuclear fraction. The wild type protein was found almost completely in the cytosol fraction after PDGF stimulation. When the cells were treated with wortmannin, most of the wild type protein was in the nuclear fraction even after PDGF stimulation. In contrast, T32A and S197A were found mostly in the nuclear fraction, and PDGF treatment did not affect the distributions of these two mutant proteins. S262A, however, was mostly located in the cytosol in resting cells, and a small portion of the protein in the nuclear fraction was eliminated on growth factor treatment. Namely, S262A behaved as the wild type FOXO4 in marked contrast to T32A and S197A. The double mutants, having a mutation at either Thr32 or Ser197, as well as the triple mutant, was mostly localized in the nuclear fraction, resembling T32A and S197A. The results indicate that phosphorylation at both Thr32 and Ser 197 is critical for the cytosolic localization of FOXO4, but that the modification reaction of Ser262 is not involved in the regulation of the intracellular distribution of this protein.

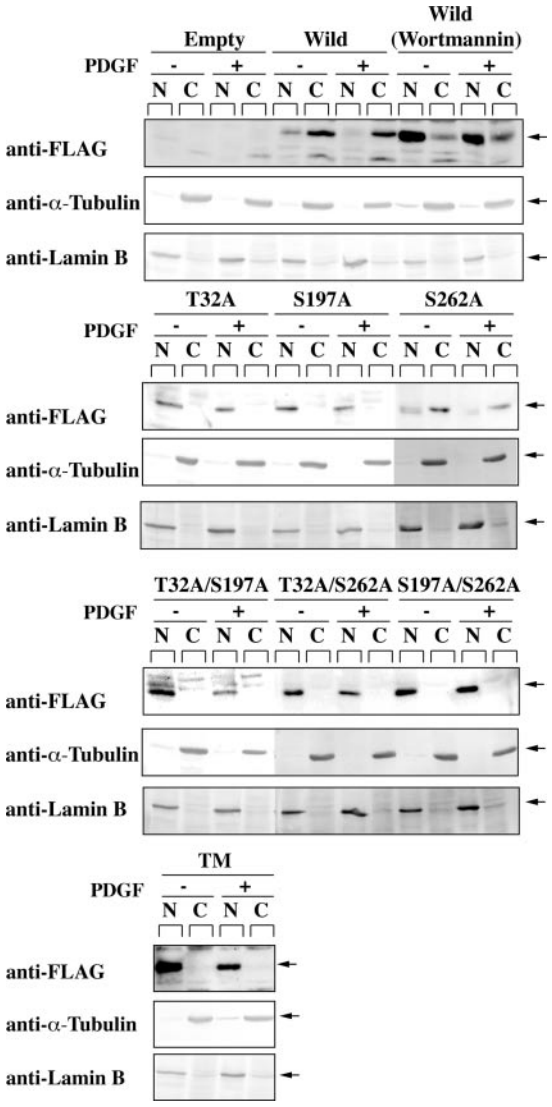

Fig. 4. **Localization of FOXO4 in NIH 3T3 cells.** FLAG-FOXO4 proteins were expressed in NIH 3T3 cells by using recombinant retroviruses. After serum starvation, the cells were incubated with (+) or without (–) PDGF, and then subjected to subcellular fractionation. Where indicated, cells expressing the wild type FLAG-FOXO4 were treated with 200 nM wortmannin for 20 min before stimulation. Equivalent amounts of nuclear (N) and cytosolic (C) fractions were subjected to immunoblot analysis with the anti-FLAG antibody, and anti–α-tubulin and anti–lamin B antibodies as markers for cytosolic and nuclear proteins, respectively. The immunoreaction was detected by means of the chemiluminescence reaction. The positions of each protein are indicated by arrows. The data are representative of three independent experiments.

Transcriptional Activity—The transcriptional activity of the FOXO4 proteins was analyzed in NIH 3T3 cells (Fig. 5). Each FOXO4 expression plasmid was transfected with reporter plasmids containing the FOXO family target sequence DBE upstream of the firefly luciferase gene (*19*). The transcriptional activity of T32A and S197A was, as in

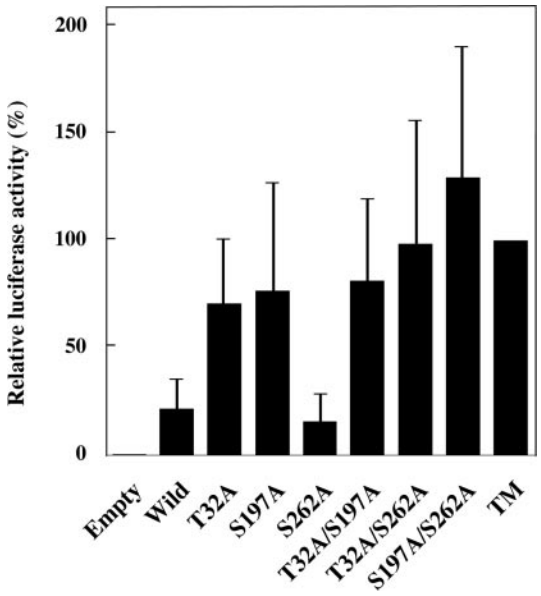

Fig. 5. **The transcriptional activity of FOXO4.** NIH 3T3 cells were co-transfected with each FLAG-FOXO4 plasmid and the reporter plasmids, cultured for 24 h, and then subjected to the reporter assay. The relative luciferase activity of the triple mutant is shown as 100%. The data represent the means ± SD for three independent experiments.

the case of the triple mutant, 3- to 5-fold higher than that of the wild type. In contrast, S262A showed similar activity to the wild type. The double mutants showed comparable activity to that of the triple mutant. These results are consistent with those as to the localization of FOXO4.

DISCUSSION

The initial studies on FOXO4, also known as AFX, had been carried out using a human clone (accession number X93996) having 501 amino acid residues with motif sites of Thr28 (T1 site), Ser 193 (S1 site), and Ser258 (S2 site) (*14*, *15*, *20*). Later, a mouse FOXO4 clone (accession number AB032770) was isolated (*19*), which has four amino acid residues at the amino-terminal end in addition to the human clone, and then a new human clone (accession number Y11284) was reported, which has an open reading frame extending in the 5′ direction with an amino-terminal end sequence homologous to that of the mouse clone (*18*). We also carried out screening of human cDNA libraries and recovered novel type clones, but no clone encoding the old 5′-end sequence was isolated. It is plausible that the old clone does not include the authentic sequence of human FOXO4, and thus the role of phosphorylation at the PKB motif sites was analyzed using a new clone having Thr32 (T1 site), Ser 197 (S1 site), and Ser262 (S2 site).

Kops *et al*. (*14*) have reported that phosphorylation at the S1 and S2 sites is critical for the regulation of FOXO4, but that the T1 site is not phosphorylated *in vitro* or *in vivo*. The present study, however, showed that PKB recognizes these three sites *in vitro*, and that FOXO4 is phosphorylated at the three motif sites *in vivo*, by phosphopeptide mapping and immunoblot analysis with phosphorylation site–specific antibodies. Analysis with mutants with the motif residues replaced by Ala indicated that the T1 and S1 sites are indispensable, whereas the S2 site is not critical, for regulation of the nuclear localization and transcriptional activity of the protein. The data obtained in the present study differ from those in the previous one (*14*), but presumably the inconsistent results were due to the use of a construct lacking the amino-terminal end sequence. Concerning the role of the S2 site, we have reported that this site is involved in the control of subcellular localization on the microscopic observation of the green fluorescent protein fused with the FOXO4 mutant of the old clone (*15*). The quantitative biochemical analysis herein ruled out a role of the S2 site in the regulation of the intracellular localization.

The properties of FOXO4 shown in this study well agree with those of FOXO1 and FOXO3. Namely, phosphorylation at the T1 and S1 sites is important for regulation of these two proteins (*11–13*, *16*, *17*), and the S2 site does not play an essential role in same cases (*12*, *13*, *16*). Furthermore, FOXO6, a new member of the FOXO family, has been identified, which has the T1 and S1 sites but the S2 site is not conserved (*28*). It is interesting to assume that the localization as well as the transcriptional activity of the FOXO family members is controlled in an analogous manner through phosphorylation at the T1 and S1 sites. For example, the S1 site is located in the nuclear localization signal sequence, and Brownawell *et al*. (*29*) have proposed that the addition of a negative charge at the S1 site reduces the affinity of the transcription factor for its nuclear import receptor and thus it remains in the cytosol. It seems possible that the association of 14-3-3 proteins with the phosphorylated T1 site, which contributes to retention of FOXO1 and FOXO 3 in the cytosol (*13*, *16*, *17*, *26*), occurs also in FOXO4. The S2 site is phosphorylated *in vivo*, and thus the modification reaction at this site may play a role in regulation of other than nuclear export in the FOXO family. It has been reported that the S1 site is initially phosphorylated and then other sites are modified in FOXO1 (*26*). The results obtained in this study suggested that PKB recognizes these three sites independently *in vivo* and *in vitro*. Precise analysis is required as to the order of phosphorylation at the PKB motif sites in the FOXO family.

In addition to PKB-mediated phosphorylation, the FOXO family members are recognized by other protein kinases. FOXO1 is constitutively phosphorylated at Ser329 by DYRK1A (dual-specificity tyrosine-phosphorylated and regulated kinase 1A) (*30*). FOXO1 is also phosphorylated at Ser322 and Ser325 by CK1 after modification at the S2 site with PKB (*31*). The Ser residues corresponding to these three phosphorylation sites in FOXO1 exist in FOXO3 and FOXO4 (*30*, *31*). On the other hand, it has been shown that the FOXO family is regulated through acetylation and deacetylation in the nucleus (*32–35*). It seems that the FOXO family members are regulated through closely related mechanisms. In contrast, the transcriptional activity of FOXO4 increases on phosphorylation at Thr451 and Thr455 (Thr447 and Thr451 in the old clone, respectively) in a Ras-Ral dependent manner (*36*), these residues not being conserved in FOXO1 and FOXO3. It is thus necessary to determine the roles of these posttranslational modification reactions in detail to clarify the roles of the transcription factors of the FOXO family.

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