FoxO6, a Novel Member of the FoxO Class of Transcription Factors with Distinct Shuttling Dynamics*

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Frank M. J. Jacobs‡, Lars P. van der Heide‡, Patrick J. E. Wijchers, J. Peter H. Burbach, Marco F. M. Hoekman, and Marten P. Smidt§

From the Rudolf Magnus Institute of Neuroscience, Department of Pharmacology and Anatomy, University Medical Center, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands

Forkhead transcription factors of the FoxO-group are associated with cellular processes like cell cycle progression and DNA-repair. FoxO function is regulated by protein kinase B (PKB) via the phosphatidylinositol 3-kinase/PKB survival pathway. Phosphorylation of serine and threonine residues in specific PKB phosphorylation motifs leads to exclusion of FoxO-proteins from the nucleus, which excludes them from exerting trans-activating activity. Members of the FoxO-group have three highly conserved regions containing a PKB phosphorylation motif. This study describes the cloning and characterization of a novel forkhead domain gene from mouse that appeared to be highly related to the FoxO group of transcription factors and was therefore designated FoxO6. The FoxO6 gene was mapped in region D1 on mouse chromosome 4. In humans, FOXO6 is located on chromosomal region 1p34.1. Embryonic expression of FoxO6 is most apparent in the developing brain, and FoxO6 is expressed in a specific temporal and spatial pattern. Therefore it is probably involved in regulation of specific cellular differentiation. In the adult animal FoxO6 expression is maintained in areas of the nucleus accumbens, cingulate cortex, parts of the amygdala, and in the hippocampus. Structure function analysis of FoxO6 compared with its group members shows that the overall homology is high, but surprisingly a highly conserved region containing multiple phosphorylation sites is lacking. In transfection studies, FoxO6 coupled to GFP showed an unexpected high nuclear localization after stimulation with growth factors, in contrast to the predominant cytosolic localization of FoxO1 and FoxO3. We also show that nuclear export of FoxO6 is mediated through the phosphatidylinositol 3-kinase/PKB pathway. Furthermore, we show using a chimeraic approach that we can fully restore the ability of FoxO6 to shuttle between nucleus and cytosol. In conclusion, the data presented here gives a new view on regulation of FoxO-function through multiple phosphorylation events and other mechanisms involved in the nuclear exclusion of FoxO-proteins.

Transcription factors of the forkhead family have an important role in development and function of an organism (1). Since the discovery of the winged helix structure (forkhead domain) in Drosophila, more than 90 genes containing the forkhead domain have been identified, in species ranging from yeast to humans (1). Daf-16, a forkhead transcription factor in Caenorhabditis elegans has been extensively studied for its role in controlling longevity and dauer formation (2). Transcriptional activity is negatively regulated via an insulin-like signal transduction cascade. In humans Daf-16 has four described orthologues, FOXO1 (FKHR), FOXO2, (AF6q21), FOXO3a (FKHR1L), and FOXO4 (AFX). Together, these proteins form the FOXO-class of forkhead transcription factors in humans. Also in mice, Daf-16 orthologues are identified and are designated FoxO1, FoxO3, and FoxO4 (3). A subset of FOXO genes has been associated with disorders like tumorigenesis and rhabdomyosarcomas. Genetic analysis of a type of acute lymphocytic leukemia revealed that the cause of the disorder is a translocation between chromosome 11 and chromosome X (4). This translocation involves fusion of the general transcription factor HTRX1 with the forkhead gene FOXO4 on the X chromosome (4). A form of rhabdomyosarcoma is caused by a translocation between chromosome 2 or chromosome 1 and chromosome 13 [t (1, 13) or t (2, 13)], which leads to fusion of the PAX7 or PAX3 gene with the forkhead FOXO1 (5, 6). The fusion product turned out to be a stronger activator compared with PAX3 or PAX7, which function as inhibitors of myogenic differentiation of migrating limb myoblasts (7). Blockage of this terminal differentiation pathway by the PAX3-FOXO1 fusion protein is the direct cause of this disorder.

Since their discovery, FOXO-members have been subject of intensive investigation, especially their place in the phosphatidylinositol 3-kinase/protein kinase B (PKB) pathway and the identification of the transcriptional targets. Binding of insulin-like substrates to the insulin receptor leads via PI3-kinase to phosphorylation and activation of PKB. As demonstrated in Drosophila, PKB phosphorylates specific motifs within FOXO proteins, inducing translocation to the cytosol, thereby preventing their transcriptional activity (8, 9).

To elucidate cellular functions of FOXO proteins, many studies focused on identification of their transcriptional targets. FOXO3a has been demonstrated to play an important part in cell cycle progression of fibroblast cells by regulating expression of the mitotic genes cyclin B and polo-like-kinase. Inter
ference with FOXO3a transcriptional activity induces defective cytokinesis, a delayed transition from M to G1, and finally accumulation of cells in the G1/M stage (10). FOXO3a triggers DNA repair through the Gadd45 protein, which was shown to be a direct transcriptional target of this forkhead protein (11). Recently FOXO3a has been shown to protect quiescent cells from oxidative stress by inducing transcription of manganese superoxide dismutase (12). Taken together, the figures indicate that FOXO proteins are of crucial importance for the ability of a cell to respond to environmental changes. Processes of proliferation, differentiation, and responsiveness to extracellular changes are highly relevant in the nervous system. The properties of FOXO proteins render them candidates to play an important role in neuronal regulatory processes. For this reason we elucidated on the identification of FOXO proteins in the central nervous system. In this study we describe the cloning and characterization of a novel member of the FOXO class and detail structural and functional properties related to gene regulation. This novel protein, FoxO6, clearly differs from FoxO1 and FoxO3 in its shuttling properties. Through mutation analysis and the generation of chimeric proteins this difference is identified as a domain absent in FoxO6 located just behind the forkhead domain in FoxO3 and FoxO1.

EXPERIMENTAL PROCEDURES

PCR, Cloning, and Sequencing—From adult C57Bl/6 mouse brain, we dissected the tissue in the ventral midbrain. Total RNA was isolated and subjected to reverse transcription-PCR for cDNA synthesis using reverse transcriptase Superscript II and both oligo(dT) and random hexamer primers. Degenerate primers (forward, 5′-GGGCTSAMHTYKSKBCAGAT-3′; reverse, 5′-TTGGTCGVRATGARTGCTTTCCA-3′) were designed to identify (novel) members of a subset of the forkhead family of transcription factors. This set of primers amplifies part of the forkhead domain of members of the FOXO group. The annealing temperature was 45°C, and PCR products were separated on a 2% agarose gel by gel electrophoresis. Fragments of the expected length of 110 bp were purified (Qiagen PCR Purification kit), ligated in pGemT Easy (Promega), and transformed to Escherichia coli DH5α. Resulting colonies were subjected to colony PCR. Fragments of appropriate length were purified (Qiagen PCR-Purification kit) and sequenced on a Beckman Coulter CEQ 2000 sequencer under standard conditions.

In Situ Hybridization—In situ hybridization was performed as follows. Cryostat sections cut at 16 μm were thaw-mounted onto Superfrost+ slides, dried, and fixed for 10 min in fresh 4% paraformaldehyde in phosphate-buffered saline. After washing with phosphate-buffered saline, sections were acetylated for 10 min in a solution containing 245 mM HCO3, 3.3 mM ammonium chloride, 458 mM NaCl, and 5.5 mM sodium acetate. Sections were washed with phosphate-buffered saline and prehybridized for 2 h in a prehybridization solution (50% deionized formamide, 5× SSC, 5× Denhardt’s solution, 250 μg/ml baker’s yeast, and 500 μg/ml sonicated salmon sperm DNA). Hybridization was performed overnight at 72°C with 400 ng/ml DIG-labeled probe added to the hybridization solution each solution was prehybridized with 2× SSC, and nesofilin was removed in 2× SSC, and sections were placed in 0.2× SSC for 2 h and washed in a solution containing 100 mM Tris/HCl, pH 7.4, 150 mM NaCl (buffer 1). Preincubation with 1.5 ml of buffer 1 with 10% heat-inactivated fetal calf serum (hiFCS) was performed for 1 h at room temperature in a humidified chamber. Sections were incubated overnight at 4°C with alkaline phosphatase-conjugated mouse anti-DIG Fab fragment (Roche), 1:5000 diluted in buffer 1 with 1% heat inactivated fetal calf serum. Sections were washed the next day in buffer 1 and equilibrated with a solution containing 100 mM Tris/HCl, pH 9.5, 50 mM MgCl2, 100 mM NaCl. Subsequently 200 μl NBT/BCIP solution (Roche) and 2.4 mg/ml final volume levamisole was added to each 100 mM Tris/HCl, pH 9.5, 50 mM MgCl2, 100 mM NaCl solution, and the color reaction was performed in the dark for about 8 h. The color reaction was stopped by adding 10 mM Tris/HCl, 5 mM EDTA, pH 8.0, and slides were dehydrated with ethanol and mounted using entellan (Merck).

RESULTS

Isolation and Characterization of FoxO6 mRNA—We used a degenerate PCR strategy to screen for (novel) members of the FOXO group of forkhead transcription factors expressed in the mouse ventral midbrain. Primers were designed to amplify part of the forkhead domain, a region with high sequence homology. Using this strategy we cloned PCR fragments encoding FoxO1, FoxO3, and FoxO4. Interestingly, we cloned a PCR fragment that showed high similarity with these genes, but differed in 10 of 110 bp compared with its closest family member. Data base analysis of this sequence in mouse genomic DNA databases led to characterization of the putative 3′ and 5′ part of a novel gene of the FOXO-group of transcription factors. Initially 7 mouse-derived ESTs from mouse tissue were identified, originating from the 3′ region (B1686281, AA656491, BFI87145, Al593097, D21486, Al425281, and BF461725), and only recently a mouse brain-derived EST was released originating from the 5′ region (CA316065). Based on genomic DNA-sequence information, primers (forward: gggcaccctagtctcctcgagcgctgattctggagcagtctgctgat

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gaagc, reverse; acttcaaccatccctcccagac) were designed to amplify the total coding region from mouse ventral midbrain cDNA. The resulting PCR fragment was cloned and sequenced. Primary sequence analysis revealed that the amplified cDNA contained a large open reading frame predicted to encode a 559 amino acid protein. The presence of a forkhead domain and overall similarity to FoxO1, FoxO3, and FoxO4, identified the protein as a novel member of the FoxO class of forkhead transcription factors (Fig. 1). Because FoxO5 is already designated in zebrafish (3), we named this gene FoxO6. Noteworthy are the recently submitted "genome scan" gene predictions (XM284000 and XM143959) based on genomic and EST sequence data. These predictions are incomplete and incorrect for the fact that part of the genomic sequence of FoxO6 is not yet present in the databases. Comparison of the deduced amino acid sequence of FoxO6, FoxO1, FoxO3, and FoxO4, demonstrated that FoxO6 is 34% identical to FoxO1, 38% identical to FoxO3, and 36% identical to FoxO4 over their shared lengths. Within the forkhead domain this identity is increased to 90% for FoxO1, 89% for FoxO3, and 90% for FoxO4 (Fig. 1).

Chromosomal Structure and Localization—In the murine genome FoxO6 is located on chromosome 4, region D1 between chromosomal markers 1283756 and X59556 (within 20 kb of marker 1283756), according to the MGSC v3 data base of the Sanger Institute. Mouse genomic data base analysis revealed that the open reading frame of FoxO6 is divided by a large intron of ~18 kb long, resulting in 2 putative exons of 414 and 1266 bp in length. A polyadenylation signal (AATAAA) is found 818 bp downstream from the stopcodon. This 3' end corresponds to 3' EST sequences, which indicates that the FoxO6 mRNA contains a 3' untranslated region of at least 818 bp long and that this is in fact the last exon of the FoxO6 gene. The startcodon (GGCGGGATACTGAG) of the mapped FoxO6 amino acid sequences lies within a proper Kozak sequence. In addition, the 5' UTR contains no upstream startcodons in either frame. These facts and the homology to FoxO1, FoxO3, and FoxO4 indicate that the mapped methionine is the correct startcodon. Based on the 5' UTR, FoxO6 contains a 5' untranslated region of at least 98 bp. Comparison of mouse FoxO6 to human genomic databases revealed that the human FoxO6 orthologue is located at chromosomal region 1p34.1. Within this regions several diseases have been mapped, but no clear indication for FoxO6 dysfunction related disease could be identified. In the human EST data base four different 3' ESTs were found, originating from brain tissue and tumor-cell lines (AI361654, AI341823, M85901, and AA927741). All human ESTs showed 95% sequence identity to mouse FoxO6.

Expression Pattern of FoxO6 in Murine Tissue—To elucidate the possible function of FoxO6 we examined the spatial and temporal expression pattern in murine tissues. In situ hybridizations using DIG-labeled probes specific for FoxO6 transcripts were performed in adult mouse brain (Fig. 2). Because FoxO5 is already designated in zebrafish (3), we named this gene FoxO6. Noteworthy are the recently submitted "genome scan" gene predictions (XM284000 and XM143959) based on genomic and EST sequence data. These predictions are incomplete and incorrect for the fact that part of the genomic sequence of FoxO6 is not yet present in the databases. Comparison of the deduced amino acid sequence of FoxO6, FoxO1, FoxO3, and FoxO4, demonstrated that FoxO6 is 34% identical to FoxO1, 38% identical to FoxO3, and 36% identical to FoxO4 over their shared lengths. Within the forkhead domain this identity is increased to 90% for FoxO1, 89% for FoxO3, and 90% for FoxO4 (Fig. 1).

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Expression Pattern of FoxO6 in Murine Tissue—To elucidate the possible function of FoxO6 we examined the spatial and temporal expression pattern in murine tissues. In situ hybridizations using DIG-labeled probes specific for FoxO6 transcripts were performed in adult mouse brain (Fig. 2). In rostral sections the FoxO6 transcript was detected in the ependyma, the medial part of the anterior olfactory nucleus, and diffuse in the cingulate cortex (Fig. 2A). More caudal, expression was detected in the shell of the nucleus accumbens, the claustrum, the dorsal endopiriform nucleus, and the cingulate cortex (Fig. 2B). Furthermore, the
transcript was detected in the posteroventral part of the medial amygdaloid nucleus, portions of the amygdalo-hippocampal area, and dorsal and ventral endopiriform nuclei.

In E12.5 embryos, a high level of expression of FoxO6 was detected in the trigeminal ganglion and tissue surrounding the lateral portion of the fourth ventricle that forms the cerebellum (Fig. 3A). The olfactory epithelium showed high amounts of the transcript, as well as the dorsal root ganglia along the embryo’s spine (Fig. 3B). Lower amounts of the transcript were found in striatal areas and in the neopallial cortex, which forms the cerebral cortex. The level of expression in the olfactory epithelium and the dorsal root ganglia was sustained in embryos of E14.5 and E18.5, whereas expression in the trigeminal ganglion was detected (D). In E15.5 embryos additional expression was detected in hippocampus (hc) and dentate gyrus (dg) (E), the thymus (th) (F), and the cortex of the kidney (ck) (G). Control experiments with sense probes of the same sequence did not give any signal.

also contains the most recently migrated cells. In the periphery, the FoxO6 transcript was detected in the thymus (Fig. 3F), the cortical region of the kidney (Fig. 3G), the whiskers and dents (data not shown). These data show that the FoxO6 gene is dominantly present in the developing and adult murine brain, indicative for a function of FoxO6 during development and in the adult functional central nervous system.

**FoxO6 Displays Distinct Nuclear Exclusion**

FIG. 2. Expression of FoxO6 in the mouse brain. In situ hybridization for FoxO6 mRNA is shown. In rostral sections (A and B) expression was detected in the medial part of the anterior olfactory nucleus (AOm), the ependyma (E), the cingulate cortex (Cg), the claustrum (Cl), the dorsal endopiriform nucleus (DEn), and the shell of the nucleus accumbens (AcbSh). More caudal (C and D) expression was detected in the medial amygdaloid nucleus (MePV), anterolateral part of the amygdalohippocampal area (AHiAL), and ventral and dorsal endopiriform nucleus (Ven and Den). Control experiments with sense probes of the same sequence did not give any signal.

FIG. 3. Expression of the FoxO6 gene in the mouse embryo. In situ hybridization for FoxO6 on embryonic mouse sagittal sections from stage E12.5 to E18.5 is shown. In E12.5 embryos (A and B), FoxO6 was expressed in the neopallial cortex (premordial cerebral cortex) (np), the trigeminal ganglion (V), the cerebellum primordium (premordial cerebellum) (cp), the olfactory neuroepithelium (on), striatum (str), and the dorsal root ganglia (drg). At E14.5 increased expression in the neopallial cortex (C), most prominent in the outer zone (oa) of the cortex is detected (D). In E15.5, the interventricular zone of neopallial cortex. In E18.5 embryos expression was detected in hippocampus (hc) and dentate gyrus (dg) (E), the thymus (th) (F), and the cortex of the kidney (ck) (G).

Noteworthy is the fact that a third Arg-Xaa-Arg-Xaa-Thr motif is found in the far C terminus of FoxO6, in a region that...
compared with other mouse FoxO members and DAF16. A

rial serines are in
serine or threonines are
FoxO1, FoxO3, FoxO4, and FoxO6. The proteins all contain the first two
C-terminally from the forkhead domain in FoxO6.

missing third conserved region containing a PKB phosphorylation site

ations of conserved regions containing putative PKB phosphorylation

positions in the forkhead domain and in the N-terminal region. Note the

 DYRK1A. The position of the forkhead domain is shown in

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Previous studies (8) in mam-

pared with FoxO1 and FoxO3

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shows no similarity to the other FoxO proteins and Daf16. In

addition, no CK1 or DYRK1A motifs are found in this region.

Therefore it is not certain whether the threonine residue in this

region is a natural substrate for PKB.

Translocation of FoxO6 Is Dramatically Decreased Compared with FoxO1 and FoxO3—Previous studies (8) in mam-

nal cell lines have shown that in response to stimulation

with insulin-like growth factors, PKB phosphorylates FOXO-

proteins. This results in translocation of the forkhead protein

from the nucleus to the cytosol (8). To test whether FoxO6

responds in a similar manner to growth factor stimulation, we

transfected HEK-293 cells with FoxO1-, FoxO3-, and FoxO6-

GFP constructs. Twenty-four hours after transfection FoxO1

and FoxO3 displayed a predominant cytosolic localization in

virtually 100% of transfected cells for FoxO1 and ~80% for

FoxO3. In strong contrast to FoxO1/FoxO3, FoxO6 was fully

localized in the nucleus 24 h after transfection (Fig. 5, 1st column).

Subsequent serum starvation for 20 h resulted in a predominant

nuclear localization for FoxO1 and FoxO3, although some cytoplasmic

fluorescence was still apparent. FoxO6 however had an exclusive nuclear localization (Fig. 5, 2nd column). When stimulated with serum, IGF-1, or insulin, FoxO1 and FoxO3 were excluded from the nucleus and showed a predominant cytosolic localization (Fig. 5, 3–5th columns). Under these conditions FoxO6 displayed a predominant nu-

clear localization. Although translocation of FoxO6 was signifi-

antly less as compared with FoxO1 and FoxO3, a general increase in cytoplasmic fluorescence was detected. This indi-

cates that some protein export from the nucleus had occurred.

Translocation of FoxO6 Is Mediated by a PI3-kinase-dep-

endent Mechanism—To assess whether nuclear export of FoxO6 is

regulated in a PI3-kinase-dependent manner, we preincubated

cells with LY294002, a PI3-kinase inhibitor, before treatment

with either IGF-1 or insulin. PI3-kinase inhibition resulted in

a significant decrease in cytosolic localization of FoxO6 in cells

treated with either IGF-1 or insulin (Fig. 6). Besides the PI3-

kinase pathway, IGF-1 and insulin can activate the MAPK

pathway as well. Cells preincubated with PD98059, an inhibi-

tor of the MAPK pathway, displayed no difference in IGF-1/

insulin-induced translocation. These findings clearly indicate

that translocation of FoxO6 upon IGF-1 or insulin stimulation

is mediated by the PI3-kinase pathway. These results are in

perfect agreement with results from similar studies done with

other FoxO proteins (16–18).

Mutation of Thr-26 or Ser-256 Blocks Nuclear Exclusion of

FoxO6—Mutation analysis in FOXO1 has shown that substi-

tution of Thr-24 or Ser-256 by alanine residues (mimicking a

non-phosphorylated state) results in a blocked nuclear exclu-

sion (16, 19). As stated before, the regions containing Thr-24

and Ser-256 in FOXO1 are highly conserved in all members.

Thr-26 and Ser-184 are the equivalent residues in FoxO6 and

are therefore potentially phosphorylated by PKB as well, re-

sulting in nuclear export. To verify this possibility, we con-

structed mutant FoxO6 proteins, where either the Thr-26 or

the Ser-184 residue was substituted by an alanine. Both muta-

tion FoxO6 proteins displayed no IGF-1-induced increase in

cytosolic localization, in contrast to the wild type protein (Fig. 7).

This clearly demonstrates that each PKB phosphorylation

motif is required for nuclear exclusion of the FoxO6 protein.

 FoxO6 Functions as a Transcription Factor—To investigate

whether FoxO6 is indeed a functional transcriptional activator,

we analyzed its activity on a luciferase reporter construct con-

taining six optimal DAF-16 binding elements (6DBE). In this

experiment we compared FoxO6 activity to FoxO1 and FoxO3
to assess whether FoxO6 functions similarly to these other two

proteins. Under the experimental conditions used, the basal

activity of the 6DBE reporter construct is very low as was found

after transfection of the EGFP vector. The data on the FoxO

activity showed that FoxO3 had the highest activity, FoxO6 activity

was intermediate, and FoxO1 showed the lowest level of

transcriptional activation (Fig. 8A). Because this experiment

was performed under serum-free conditions the localization of

different factors is mainly nuclear. Previous studies (20)

have shown that FoxO transactivation is highly dependent on

the phosphorylation status of the serine located in the DNA

binding domain. Phosphorylation of this serine is described to

reduce DNA binding and thereby transactivation (20). FoxO6

has a nuclear localization under serum and serum-free condi-
tions. Therefore, we compared FoxO6-WT and Ser-184 mutants

to investigate possible differences in transactivation as a con-

sequence of the phosphorylation state of Ser-184 (Fig. 8B).

FoxO6-WT had low transactivation properties as compared

with the FoxO6-Ala-184 under serum conditions and could be

reduced even further by mutating FoxO6-Ser-184 to an aspar-

cic acid (FoxO6-Asp-184). Serum starvation increased

FoxO6-WT activity to a comparable level as found for the

FoxO6-Ala-184 mutant. These data indicate that indeed the

phosphorylation state of Ala-184 is essential in regulating the

transactivating properties of FoxO6 independent of its subcel-
lular localization.

Shutting of FoxO6 Can Be Restored through Insertion of a

FoxO3-derived Phosphorylation Domain—Here we address

why FoxO6 is largely retained in the nucleus after growth

factor stimulation in contrast to FoxO1 and FoxO3. In our view,

there are two structural differences that potentially underlie
FoxO6's remarkable distinct translocation efficiency (Fig. 9). The first domain is a nuclear export signal (NES) described by Brunet et al. (21). They report that in FOXO3a, two NESs are crucial for nuclear export. FoxO6 has a putative (optimal) NES corresponding to the first in FOXO3 but lacks convincing similarity to the second NES. The second domain that is absent in FoxO6 as compared with the other FoxO proteins, as mentioned above, contains a stretch of four serine residues, which have been shown to be phosphorylated by the kinases PKB, CK1, and DYRK1A. Mutational analysis of FOXO1 by Rena et al. (14) revealed that mutation of Ser-319, Ser-322 or Ser-319/Ser-322/Ser-325/Ser-329 to alanine residues results in a decreased speed of nuclear export. To test whether the absence of a second NES or the stretch of phosphorylation sites could explain the impaired nuclear export of FoxO6, we constructed two chimeric proteins (Fig. 9). In the first chimeric protein, FoxO6[NES2], we replaced part of FoxO6 for the FoxO3 sequence containing the second NES. In the second chimeric protein, FoxO6[4Ser], part of the FoxO6 sequence was replaced by the domain containing the stretch of serine residues as present in FoxO3. Both chimeric constructs were transfected and localization was monitored at three different stages: 24 h after transfection, deprived of serum for an additional 20 h, and subsequent insulin stimulation. The subcellular localization of FoxO6[NES2] was similar to wild type FoxO6. (Fig. 10A, 2nd and 3rd rows). Interestingly, localization of FoxO6[4Ser] was significantly altered compared with wild type FoxO6. After transfection, cells showed both nuclear and cytosolic FoxO6[4Ser]. This resembled FoxO3 localization rather than FoxO6 (Fig. 10A, 1st, 2nd and 4th rows). Serum starvation led to an exclusive nuclear localization of FoxO6[4Ser] and subse-
quent insulin stimulation resulted in a surprisingly high level of cytosolic staining in all cells. Insulin induced FoxO6\[4Ser\] cytosolic relocation was significantly reduced by PI3-kinase inhibition, indicating that nuclear export is PI3-kinase-mediated. In addition, we monitored cellular relocalization upon insulin stimulation in combination with leptomycin B, an inhibitor of Crm1- and NES-dependent nuclear export (Fig. 10B) (21). The data from this experiment showed that FoxO6\[4Ser\] was exclusively localized in the nucleus, indicating that insulin-stimulated nuclear export of the FoxO6\[4Ser\] chimeric protein is a Crm1- and NES-dependent process.

Data obtained from the insulin-induced relocalization to the cytosol was quantified by counting cells on a representative area of the slide and monitoring FoxO localization in each individual cell (Fig. 11). FoxO3 was present in about 50% of the cells exclusively in the nucleus (Fig. 11, N) and in about 50% both in the nucleus and the cytosol (Fig. 11, C/N) under serum-free conditions. After the application of insulin this shifts largely (~50% of the cells) to an exclusive cytosolic localization (Fig. 11, C). FoxO6 was almost exclusively localized in the nuclear compartment under serum-free conditions and was present in about 20% of the cells both in the nucleus and in the cytosol (20% C/N) after insulin treatment. No clear differences between the FoxO6\[NES2\] chimera and the wild type FoxO6 protein was observed. Under serum-free conditions, the FoxO6\[4Ser\] mutant matched wild type FoxO6 but showed a dramatic shift toward an exclusive cytosolic localization after insulin treatment. In sharp contrast to the wild type FoxO6, the FoxO6\[4Ser\] chimera was exclusively located in the cytosol in ~30% of the cells.

**DISCUSSION**

Here we describe the identification, expression, and functional characterization of a novel gene of the FoxO group of forkhead transcription factors in mouse, which we designated FoxO6. *In situ* hybridization experiments showed that the FoxO6 gene was predominantly expressed in a restricted manner in the developing and adult brain, especially cortical and hippocampal structures. In the adult brain, besides some positive cells scattered through the cerebral cortex, cortical expression was limited to the endopiriform nuclei. The endopiriform nucleus is a limbic related structure and is thought to play a part in the acquisition of conditioned fear (22) and is associated with temporal lobe epileptogenesis (23). It has been reported that this cortical structure is connected to the claustrum, amygdala, and the anterior olfactory nucleus (23). Connections between the endopiriform nucleus and hippocampal structures were found as well. Interestingly, FoxO6 is expressed in all these structures in the adult animal, suggesting that FoxO6 expression is consistent with a functional neuronal network.

The similarity of FoxO6 to the other members of the FoxO class, FoxO1, FoxO3, and FoxO4, was highest in the forkhead domain, whereas other regions were highly to moderately conserved. The homology is remarkable in two regions that have been reported to be involved in phosphorylation by PKB via the PI3-kinase/PKB pathway. Strikingly, a third conserved region containing a stretch of four phosphorylation sites as found in the other FoxO proteins, including Daf16, is not present in FoxO6. Furthermore, in FoxO6 there is reasonable sequence
conservation in regions implicated in transactivation as described by So and Cleary (24). With the use of a reporter construct we have shown that FoxO6 is indeed a fully functional transcription factor that can be compared with FoxO1 and FoxO3 in its transactivating property. Experiments showed that there are differences in transcriptional activity among individual FoxO proteins.

To investigate possible differences in translocation kinetics between FoxO6 and other FoxO proteins, we included FoxO1 and FoxO3. Our data show that FoxO1 and FoxO3 are mainly located in the cytoplasm under serum conditions, whereas FoxO6 was mainly located in the nucleus. The high nuclear level of FoxO6 could imply transcriptional activity under these conditions, in contrast to FoxO1 and FoxO3. Serum starvation resulted in a predominant nuclear localization of all FoxO proteins. Subsequent stimulation with growth factors resulted in a predominant cytosolic localization of FoxO1 and FoxO3 proteins, which is conform data from other groups (16, 17). Although the cytosolic level of the FoxO6 protein was slightly increased after stimulation, indicating the ability of FoxO6 to translocate, FoxO6 protein was still predominantly localized in the nucleus. Apparently, some intrinsic property of FoxO6 significantly influences the quantity or ratio between nuclear import and export.

To address the structural properties of FoxO6 that underlie the distinct translocation properties, we first determined whether the PKB motifs in FoxO6 are functionally conserved and whether the observed translocation was mediated through the PI3-kinase/PKB pathway. In all FoxO proteins, including FoxO6, two regions containing a PKB phosphorylation motif are conserved. In FOXO1, PKB-catalyzed phosphorylation of Ser-256 is thought to be critical for phosphorylation of Thr-24. These phosphorylated amino acids form a motif for binding of 14-3-3 proteins that in turn stimulate nuclear exclusion and cytoplasmic retention by masking a nuclear localization signal (19, 21, 25). Inhibition of PI3-kinase, or mutation of these PKB phosphorylation motifs in FOXO1, results in a blocked nuclear exclusion (19). These studies clearly indicate that PI3-kinase/PKB-mediated phosphorylation of these residues is critical for translocation of the forkhead protein from nucleus to cytosol. In this study we show that this is also the case for FoxO6. We showed that blockade of PI3-kinase or mutation of Thr-26 or Ser-184 all cause the inhibition of FoxO6 nuclear export. This indicates that the regions containing Thr-26 and Ser-184 in FoxO6 are indeed functionally conserved PKB phosphorylation sites.

The subcellular localization of FoxO6 is mainly nuclear and can be influenced mildly by growth factor stimulation. The transactivation capacities are however very sensitive to the presence of growth factors. Under growth factor-deprived conditions the wild type FoxO6 protein has comparable activity to the FoxO6-Ser-184 to alanine mutant protein, which cannot be phosphorylated in its DNA binding domain and thus has constitutive DNA-binding activity. The FoxO6-Asp-184 mutant protein, which mimics the phosphorylated state, did also display increased transactivational activity after growth factor deprivation but still far less compared with the wild type FoxO6 protein. This can be explained by a reduction in DNA-
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binding activity as is shown extensively for FOXO1 (20). Interestingly, under growth factor conditions the wild type FoxO6 protein still has the capacity to transactivate, which is probably a direct result of its prominent nuclear localization. Although FoxO6 is mainly nuclear localized its transcriptional activity is still tightly regulated, probably through phosphorylation of the Ser-184.

Two domains implicated in nucleo-cytoplasmic shuttling in all known FoxO proteins are absent in FoxO6. The first domain is a region that functions as a NES as present in the C-terminal part of FOXO3 (21). The second domain is the stretch of four phosphorylation sites just downstream of the forkhead domain, highly conserved in all other FoxO-members. Recent findings with FOXO1 concern the PKB-catalyzed phosphorylation of serine 319 in this region. This event primes CK1-mediated phosphorylation of serine residues 322 and 325 (14). Serine residue 329 in FOXO1 is a substrate for phosphorylation by DYRK1A in a PI3-kinase-independent manner (15). Studies using FOXO1 with artificial mutations in this conserved region show that substitution of Ser-319 and Ser-329 by alanine residues results in an increased nuclear localization in the absence of growth factors (15, 19). Furthermore, phosphorylation of Ser-319, Ser-322, and Ser-325 together with Ser-329 form an acidic patch that functions as a NES (14). Indeed, mutation analysis of FOXO1 by Rena et al. (14) revealed that mutation of Ser-319, Ser-322, or Ser-319/Ser-322/Ser-325/Ser-329 to alanine results in a decreased speed of nuclear export. The same study shows no decrease in speed of nuclear export for the Ser-329 to alanine mutant.

In the present study, using an artificial chimeric FoxO6 protein, we succeeded in re-establishing nuclear export to a level comparable with FoxO3. The chimeric protein FoxO6[4Ser] consists of FoxO6 in which we replaced part of the gene for the corresponding part of FOXO3 containing four phosphorylation sites as described above. This chimeric protein was highly comparable with FoxO6 in its overall localization. Another chimeric protein FoxO6[NES2] in which we placed the second FoxO3 NES domain showed no significant changes in cytoplasmic relocation. These results clearly indicate that the absence of an important functional domain in FoxO6 results in a dramatically reduced nucleo-cytoplasmic shuttling. The consequential distinct localization of FoxO6 suggests a different timing and/or duration of transcriptional activity for this transcription factor. The data presented here widen the understanding of the regulation of subcellular relocation of FoxO proteins and their transactivating potential, which may provide new insights in FoxO mediated processes.

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Frank M. J. Jacobs, Lars P. van der Heide, Patrick J. E. C. Wijchers, J. Peter H. Burbach, Marco F. M. Hoekman and Marten P. Smidt

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