A Winged Helix Forkhead (FOXD2) Tunes Sensitivity to cAMP in T Lymphocytes through Regulation of cAMP-dependent Protein Kinase R1α*

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Forkhead/winged helix (FOX) transcription factors are essential for control of the cell cycle and metabolism. Here, we show that spleens from Mf2−/− (FOXD2−/−) mice have reduced mRNA (50%) and protein (35%) levels of the R1α subunit of the cAMP-dependent protein kinase. In T cells from Mf2−/− mice, reduced levels of R1α translates functionally into 2-fold less sensitivity to cAMP-mediated inhibition of proliferation triggered through the T cell receptor-CD3 complex. In Jurkat T cells, FOXD2 overexpression increased the endogenous levels of R1α through induction of the R1α1b promoter. FOXD2 overexpression also increased the sensitivity of the promoter to cAMP. Finally, co-expression experiments demonstrated that protein kinase B or Akt1 work together with FOXD2 to induce the R1α1b promoter (10-fold) and increase endogenous R1α protein levels further. Taken together, our data indicate that FOXD2 is a physiological regulator of the R1α1b promoter in vivo working synergistically with protein kinase B to induce cAMP-dependent protein kinase R1α expression, which increases cAMP sensitivity and sets the threshold for cAMP-mediated negative modulation of T cell activation.

In T cells, activation of the cAMP-dependent protein kinase type I (PKA type I)1 pathway inhibits the downstream activation events that follow TCR triggering (1). PKA type I is recruited to the TCR-CD3 complex following T cell activation (2) and elicits an inhibitory pathway in lipid rafts that negatively modulates signaling through the TCR. This pathway involves activation of C-terminal Src kinase by PKA type I. Active C-terminal Src kinase subsequently inhibits Lck by phosphorylation of the C-terminal regulatory tyrosine 505 (3). This inhibitory mechanism determines the threshold of T cell activation with implications in several disease conditions. In human immunodeficiency virus infection, high levels of cAMP and thereby hyperactivation of PKA type I leads to T cell hyporesponsiveness and T cell anergy (4, 5). Conversely, a deficiency in PKA type I regulatory subunit expression and PKA type I activity has been reported in the autoimmune disorder systemic lupus erythematosus (6).

PKA is activated by the intracellular second messenger cAMP, leading to dissociation of two active catalytic (C) subunits from a regulatory dimer (R2) (reviewed in Ref. 7). The R subunit isoforms (R1α, R1β, R1α1, and R1ββ) can combine with the catalytic subunit to form either PKA type I (R1α1βC2/RIββC2) or PKA type II (R1α1αC2/RIββC2) holoenzymes that have different affinities for cAMP and thus activate at low or high local concentrations of cAMP in the cell (K1/2 of 50–100 nM versus 200–400 nM, respectively) (8). PKA types I and II are also targeted differently in the cell through binding to A kinase-anchoring proteins (reviewed in Ref. 9). When R1 subunits are up-regulated, cAMP sensitivity of PKA increases and thereby lowers the threshold for activation of cAMP-mediated downstream effects (10). Approximately 75% of PKA in T cells is of type I, and R1α is the dominating R isoform.

A complex set of mechanisms appears to regulate expression of the R1α subunit of PKA. First, several promoters differentially regulate the expression of alternatively spliced R1α mRNAs. Second, these mRNAs contain different 5′-untranslated regions encoded by the first exons that may exhibit different translation efficiency. Five R1α splice variants have been cloned (R1α1–e), two of which are ubiquitously expressed (R1α1a and R1α1b) (11–13). In addition, R1α protein levels are regulated by mechanisms such as rapid degradation of excess protein not associated with catalytic subunit, resulting in a regulatory:catalytic ratio of ~1:1 (14). R1α expression is regulated by cAMP in many cell types, and regulation at both the transcriptional and post-transcriptional level has been reported (12, 15, 16).

Winged helix/forkhead transcription factors FOXD1 (Frea4) (17, 18) and FOXD2 (Frea9/FKHL17) (19) were both cloned from human kidney and share identical DNA-binding domains but have very low similarity in other regions. We have recently reported that FOXD1 and the related factor FOXC2 function as inducers of R1α expression in testicular Sertoli cells and adipocytes, respectively (21, 22). Having discovered the presence of FOXD2 in T cells, we wanted to examine whether FOXD2 functions as an inducer of R1α expression in T cells by a similar mechanism as reported in adipocytes and testicular cells.

This paper is available on line at http://www.jbc.org

1 The abbreviations used are: PKA, cAMP-dependent protein kinase; TCR, T cell receptor; wt, wild type; PKB, protein kinase B; CMV, cytomegalovirus; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propane sulfonate; RT, reverse transcription; CAT, chloramphenicol acetyltransferase.

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FoxD2 is expressed in leukocyte subsets. Total cellular RNA from Jurkat cells and from purified human peripheral blood B cells, T cells, and monocytes was reversed transcribed using both oligo(dT) and random hexamers. Subsequently, the cDNA was subjected to 45 PCR amplification cycles using primers within the coding region of human FOXD2. The cDNA was followed by separation and visualization of amplification products on 1% agarose gels stained with EtBr. Relative β-actin levels in the different leukocyte subset samples determined by real time RT-PCR are presented below. Nd, not determined.

Here, we report the finding that T cells from FOXD2−/− mice have reduced levels of Rl1b mRNA and Rl1 protein and a decreased sensitivity to cAMP-mediated inhibition of immune function. Furthermore, FOXD2 regulates the Rl1b promoter and elevate endogenous levels of Rl1 protein. In conclusion, our data indicate that FOXD2 is important for expression of RNA and Rl protein in T lymphocytes in vivo and thereby tunes the sensitivity to cAMP and sets the threshold for cAMP-mediated immunomodulation.

EXPERIMENTAL PROCEDURES

Reverse Transcription (RT)-PCR—Complementary DNA was synthesized by reverse transcription using a commercial kit (Invitrogen, ThermoScript™ RT-PCR System, catalog number 1146-024). Total cellular RNA (5 μg of Jurkat, T cell, B cell, or monocyte total RNA) was reverse transcribed at 42 °C using both oligo(dT) and random hexamer primers. Subsequently, the cDNA was subjected to 45 PCR amplification cycles (95 °C for 30 s, 99 °C for 10, 59 °C for 1 min, and 70 °C for 2 min) and a final incubation at 72 °C for 10 min. Amplification products were separated on a 1% agarose gel stained with ethidium bromide. The human FOXD2 sense 5’-GGAGCACAGCGGCATGTCG-3’ and antisense 5’-CGATGTTACCAGAGGAACTTTGC-3’ primers amplified a 197-bp fragment of the FOXD2 cDNA.

Real Time Quantitative RT-PCR—Sequence-specific PCR primers were designed for β-actin (forward primer, 5’-AGGCACAGCGGCTGTCG-3’ and reverse primer, 5’-TCTCCGACTGTCGAGCTAT-3’; GenBank™ accession number NM_001101) using the Primer Express software version 1.5 (Applied Biosystems, Foster City, CA). Quantification of mRNA was performed using the ABI Prism 7700 (Applied Biosystems) as suggested by the manufacturer. Briefly, 100 ng of RNA was reverse transcribed using TaqMan RT reagents (Applied Biosystems). SyBr Green assays were performed using 2 μl SyBr Green Universal Master Mix (Applied Biosystems) and 300 nm sense and antisense primers and water made up to 25 μl. The specificity of the SyBr Green assays was assessed by melting point analysis and gel electrophoresis. All of the samples were analyzed in triplicate.

RNA Extraction and Northern Blot Analysis—Total RNA from homogenized mouse spleens was extracted by the guanidium isothiocyanate/CeCl method as previously described (23), and Northern blot analysis was performed using 20 μg of total RNA. The RNA was denatured in 50% (v/v) formamide and 6% (v/v) formaldehyde, subjected to electrophoresis in a 1.5% (v/v) agarose gel containing 6.7% formaldehyde, and blotted onto a nylon membrane. Complementary DNA probes for mouse Rl1b (11) and human Rl (12) cDNAs were labeled with [32P]dCTP using the megaprime DNA labeling system (Amersham Biosciences). Hybridization was performed with 50% formamide at 42 °C, and the filters were washed four times in a solution containing 0.1–0.5× SSC (300 mM NaCl and 30 mM sodium citrate, pH 7.0) with 0.1% SDS at 50 °C for 30 min. The filters were subjected to electronic autoradiography in a Packard Instant Imager β-scintillation counter.

Immunoblotting—Jurkat cells and C57BL/6 mouse spleen lymphocytes (splenocytes) were collected by centrifugation (10 min, 600 × g), washed once in cold phosphate-buffered saline, and resuspended in 500 μl of a buffer containing 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 10 mM CHAPS (Sigma) and Complete™ protease inhibitor mix (1 tablet/10 ml; Roche Applied Science). Cell suspensions were sonicated three times (Heat Systems Ultrasonic) and centrifuged for 5 min at 12,000 × g. The supernatants were stored at −70 °C until analysis. The protein samples were diluted in SDS sample buffer and denatured for 5 min at 100 °C before loading onto a one-dimensional SDS-polyacrylamide gel (4.5% stacking gel, 10% separating gel). 20 μg of total protein were loaded in each lane, subjected to electrophoresis, and subsequently transferred onto polyvinylidenefluoride membranes (Millipore, Bedford, MA) by electroblotting. The membranes were blocked overnight at 4 °C in a solution containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 5% milk and then incubated with a mouse monoclonal antibody against human or rat Rl and Rl (1:250) (Transduction Laboratories, Lexington, KY) or a rabbit polyclonal antibody against catalytic subunit 1 (1:250) (C-20, Santa Cruz Biotechnol-ogy Inc., Santa Cruz, CA) in blocking solution for 2 h at room temperature. The membranes were washed in a solution containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20. Immunoreactive proteins were visualized by Supersignal ( Pierce) using horseradish peroxidase-conjugated mouse or rabbit secondary antibodies (1:5000) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and subjected to autoradiography. The films were scanned, and the densities of bands were quantified using the Scion Image package (downloaded from www.scioncorp.com).

RESULTS

FOXD2 Is Expressed in T Cells—The presence of FOXD2 in leukocytes was initially seen in a tissue blot screen (not shown), and subsequently transfected on polyvinylidenefluoride membranes (Millipore, Bedford, MA) by electroblotting. The membranes were blocked overnight at 4 °C in a solution containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20. Immunoreactive proteins were visualized by Supersignal (Pierce) using horseradish peroxidase-conjugated mouse or rabbit secondary antibodies (1:5000) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and subjected to autoradiography. The films were scanned, and the densities of bands were quantified using the Scion Image package (downloaded from www.scioncorp.com).

Mouse Splenocyte Promoter Assays—Spleens from adult C57BL/6 FOXD2−/− (24) or wt mice were extracted and converted into single-cell suspensions by squeezing through a cell strainer in serum-free medium (RPMI 1640 medium, 1% non-essential amino acids, 2 mM t-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin/streptomycin). Erythrocytes were depleted in a buffer containing 10 mM KOH, 155 mM NH4Cl, and 8.9 μM EDTA. Spleen lymphocytes (2.5 × 106/ml) suspended in the medium defined above supplemented with 10% heat-inactivated fetal calf serum were seeded into anti-mouse CD3 T cell activation plates (Becton Dickinson) in a total volume of 100 μl and placed in a CO2 incubator at 37 °C for 48 h. After 48 h of culturing, the cells were pulsed with 5 μCi of [3H]thymidine, and 4 h later cultures were harvested onto filter plates and subsequently analyzed by β-scintillation counting. When used, CAM agonist (8-CPT-cAMP; 0–100 μM; BioLog, Life Science, Bremen, Germany) was added prior to incubation.

Construction of Reporter and Expression Plasmids—Deletion constructs from the Rl1a promoter region Rl1a1b+1b (−882 to +77), Rl1a1b (−882 to −310), and Rl1b (−406 to +77) were inserted into the pCAT basic reporter vector (12). The numbers are relative to the downstream transcription start of promoter 1b. The expression vector for FOXD2 was constructed by insertion of the coding region into pCB6 + , and the FOXD1 coding region was inserted in pEVFR. Expression vectors for wt or mutant PKB/Akt1 isoforms were created in pCMV6 fused to a C-terminal hemagglutinin epitope tag (25). All of the expression vectors contained CMV promoters. The internal luciferase control vector was pGL3 control (Promega).

Preparation, Stimulation, and Transfection of Cell Cultures for Promoter Analyses—Jurkat TAg cells (a human leukemic T cell line stably transfected with SV40 large T antigen) were grown in RPMI 1640 medium (Invitrogen) containing streptomycin (100 μg/ml), and penicillin (75 μg/ml) which were supplemented with 10% heat-inactivated fetal calf serum. For transfections, 2 × 105 cells were resuspended in 0.4 ml of Opti-MEM (Invitrogen) and transiently transfected with a total of 20 μg of DNA (2.5 μg of CAT reporter plasmid, 2 μg of internal luciferase control vector, 7.5 μg of expression vector(s) for FOX and/or PKB, and the respective empty vectors to a total of 20 μg) by electroporation (250 V/cm in a microfarad) in 25 μl in 4 μl with a 0.4-cm gap. The cells were then expanded in 20 ml of complete medium in 75-cm2 culture flasks and incubated for 20 h. For stimulation, the CAM agonist 8-CPT-cAMP was added to a final concentration of 100 μM after 20 h, and the cells were cultured for another 10 h.

Luciferase and CAT Assays—The Jurkat cells were harvested in radioactivity buffer (Promega) 2 h after transfection. CAT activities were measured using an organic phase extraction method (26) and normalized for expression from the Luciferase-encoding vector pGL3 Control (Promega). The luciferase activities were measured using a luciferase assay reagent (Promega) and a Wallac 1251 luminometer (Amersham Biosciences, Helsinki, Finland).

FIG. 1. FOXD2 is expressed in leukocyte subsets. Total cellular RNA from Jurkat cells and from purified human peripheral blood B cells, T cells, and monocytes was reversed transcribed using both oligo(dT) and random hexamers. Subsequently, the cDNA was subjected to 45 PCR amplification cycles using primers within the coding region of human FOXD2. The cDNA was followed by separation and visualization of amplification products on 1% agarose gels stained with EtBr. Relative β-actin levels in the different leukocyte subset samples determined by real time RT-PCR are presented below. Nd, not determined.
antibodies against RI mRNA (mice (6)). These observations indicate that FOXD2 of the RI mRNA was not detected (not shown). Western blotting showed that spleen lymphocytes deficient in FOXD2 were less sensitive to cAMP than wt spleen lymphocytes as illustrated by the bar chart above. Reduced RIα mRNA and protein expression in FOXD2−/− mice. A and B, Northern blot analysis was performed using total RNA from homogenized spleens from either homozygous (−/−) (black bars) or heterozygous (+/−) (light gray bars) FOXD2 mutant and wt (+/+)(gray bars) mice (n = 2 in each group). The filters were hybridized with 32P-labeled probes for RIα CDNA (A) and mouse RIα 1b 5′-untranslated region (1b) (B). The levels of RIαα and RIα 1b mRNA were analyzed by densitometric scanning, normalized for 28 S RNA levels, and presented relative to wt set to 1 (bar chart). The elevated levels of RIα mRNA in FOXD2−/− mice are due to overloading in lane 3. C, 20 μg of total protein from FOXD2−/− (black bar) (n = 3) and wt (light gray bar) (n = 2) spleen lymphocyte whole cell lysates were examined by immunoblotting using antibodies against RIα and RIαα. Relative levels of total protein were assessed by Coomassie staining of the blots and densitometry. The levels of RIα and RIαα protein are normalized for total protein levels and depicted as intensity relative to wild type in the bar chart above.

Reduced Levels of RIα mRNA and Protein in Spleens from FOXD2−/− Mice—To examine whether FOXD2 regulates RIα expression in T cells by a similar mechanism as FOXC2 in adipocytes (22) and FOXD1 in testicular Sertoli cells (21), we first examined spleens from homozygous (−/−) and heterozygous (+/−) FOXD2 null mutant mice compared with wt (+/+ ) for levels of expression of total RIα mRNA (Fig. 2A) and levels of the RIα 1b mRNA with the 1b leader exon originating from the RIα 1b promoter (Fig. 2B). We found that levels of both total RIα mRNA and of the 1b splice variant were reduced by 40–50% in the FOXD2-deficient mice relative to wt, whereas RIα 1a mRNA was not detected (not shown). Western blotting showed that RIα protein levels were decreased by 35% in FOXD2−/− compared with wt spleens, whereas RIαα levels were increased more than 2-fold (Fig. 2C). These observations indicate that FOXD2 regulates RIα mRNA and protein levels in T lymphocytes in vivo.

FOXD2 Tunes the Sensitivity of T Lymphocytes to cAMP in Vivo—To explore the impact of FOXD2 regulation of RIα on cAMP-mediated inhibition of T lymphocyte function in vivo, FOXD2−/− and wt spleen lymphocytes were activated by anti-CD3 and subjected to lymphocyte proliferation assays in the absence and presence of 8-CPT-cAMP at increasing concentrations (0–100 μM). The data from proliferation assays showed that spleen lymphocytes deficient in FOXD2 were less sensitive to cAMP than wt spleen lymphocytes as illustrated by the right-shifted inhibition curve in Fig. 3A. When examining four null mutant and three wt mice it was evident that T cells from all the mutant mice were less sensitive to inhibition of T cell function by cAMP than wt littermates as illustrated by the increase in IC50 values for cAMP inhibition of T cell proliferation in the FOXD2−/− mice (Fig. 3B).

FOXD2 Increases the RIα 1b Promoter and Increases the Sensitivity of the Promoter to cAMP—We next transfected Jurkat T cells with pCAT reporter constructs containing either the RIα 1a, the RIα 1b, or the total RIα 1a+1b promoter region (Fig. 4A) together with expression construct for FOXD2 or the related factor FOXD1 (Fig. 4B). Reporter activity directed by constructs containing the RIα 1a+1b and RIα 1b promoter, but not the construct containing the upstream RIα 1a promoter alone, was induced 2–4-fold by both FOXD2 and FOXD1. A construct containing the RIα 1a promoter with the addition of 1.8 kb of 5′-flanking sequences showed no further effect on reporter expression or induction by FOXD2 (not shown). However, elements immediately upstream of RIα exon 1a appeared to induce basal activity of the RIα 1b promoter. Elevating the amounts of FOXD2 expression vector up to 12-fold relative to the RIα 1b reporter construct demonstrated that maximal induction of the RIα 1b promoter by FOXD2 was at least 5.8-fold (Fig. 4C). However, we observed no more than 2-fold induction by introducing the corresponding amount of FOXD1 expression vector, indicating that FOXD1 and FOXD2 share the same function but display different efficacy in Jurkat cells. We next explored whether expression of FOXD2 affected the responsiveness of the RIα 1b promoter to cAMP and transfected the RIα 1b reporter vector together with the expression vector for FOXD2 or the corresponding empty vector (Fig. 4D). The cells were treated with 100 μM 8-CPT-cAMP for 10 h or left untreated, which produced a 2-fold induction of the RIα 1b promoter in the presence of endogenously expressed FOXD2, but this induction was increased to 5-fold in cells overexpressing FOXD2, suggesting that FOXD2 partly mediates the effect of cAMP in activating the RIα 1b promoter. Similar results were obtained using the RIα 1a+1b construct as a reporter, whereas the RIα 1a reporter was not affected (data not shown).

Co-expression of PKBα Enhances the Effect of FOXD2 on RIα Expression—The inhibitory effect of PKBα on the FOXO subfamily of forkhead transcription factors (FKHR, FKHR-L1, and
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Fig. 3. Reduced sensitivity of FOXD2−/− spleen lymphocytes to cAMP. Spleen lymphocytes from FOXD2−/− and wt mice were activated by CD3 in the absence and presence of increasing concentrations of 8-CPT-cAMP (0–100 μM). After 48 h of culture, the cells were pulsed with 5 μCi of [3H]thymidine, and 4 h later cultures were harvested onto filter plates and subsequently analyzed by β-scintillation counting. A, data are shown as proliferation at various concentrations of cAMP relative to untreated cells (mean ± S.E.). IC₅₀ values for FOXD2−/− (empty circles) and wild type (filled circles) are indicated. One representative experiment is shown. B, IC₅₀ values (mean ± S.E.) obtained from four FOXD2−/− (black bar) and three wt (light gray bar) mice subjected to proliferation assays in the presence of increasing concentrations of cAMP as in A.

Fig. 4. FOXD2 induces expression from the RIIb promoter and is implicated in mediating the sensitivity of the promoter to 8-CPT-cAMP. A, schematic depiction of the RIIa promoter constructs used in this study: RIIa + 1b (−882 to +77), RIIa + 1b (−406 to +77), inserted upstream of the CAT reporter gene. B, 2.5 μg of promoter constructs RIIa + 1b, RIIa, and RIIb or empty vector were transfected into Jurkat TAg cells (20 × 10⁶) by electroporation, together with 7.5 μg of expression vectors for FOXD2 (gray bars), FOXD1 (light gray bars), or the corresponding empty expression vectors (black bars), as well as a luciferase expression vector (pGL3Control, 2.0 μg) as an internal control. The cell cultures were harvested after 20 h and assayed for CAT and Luc activities. C, the RIIb construct (1.0 μg) was transfected together with luciferase control vector (2 μg), 4–12 μg of FOXD2 expression vector or 12 μg of FOXD1 expression vector, and empty vector to a total of 20 μg. D, cells transfected with the RIIa + 1b reporter construct (2.5 μg) and 7.5 μg of FOXD2 expression vector (right 2 bars) were treated with 8-CPT-cAMP (100 μM) for 10 h (light gray bars) or left untreated (black bars). The data show reporter activities normalized for expression of the internal luciferase reporter and represent the means ± S.E. of three separate transfections (B and D) or half-range (C). Activity of the RIIb reporter construct in the absence of FOXD2 expression and cAMP was assigned the value of 1.

AFX) is well documented. Furthermore, we have recently reported that PKBα enhances the effect of a FOXD family member, FOXD1, on the RIIb promoter in testicular Sertoli cells but not in 3T3-L1 adipocytes (21). To investigate whether PKBα is involved in FOXD-mediated activation of RIIb in T cells, we transfected Jurkat T cells with 2.5 μg of pCAT reporter constructs containing the RIIa + 1b, 1a, or 1b promoters together with expression vector for FOXD2 and/or PKBα (Fig. 5A). Although FOXD2 overexpression did not induce the RIIa + 1b promoter more than 1.4-fold in these experiments, we found that the combination of FOXD2 and PKBα induced the RIIa + 1b promoter 10-fold, whereas PKBα alone induced expression of the RIIa + 1b promoter 5-fold, indicating that PKBα may influence RIIa expression through endogenous FOXD2 or by alternative mechanisms in these cells. When FOXD1 and PKBα were co-transfected in Jurkat cells, a 6.3-fold induction of the RIIa + 1b promoter was observed (Fig. 5B). To assess the importance of the localization of PKBα for induction of RIIa expression, we examined the effect of a myristylated PKBα mutant compared with a wt kinase (Fig. 5C). Myristylated PKB
is reported to be constitutively membrane-bound and constitutively active (21, 25). We observed that membrane-restricted PKBα alone had a modest inductive effect on the basal expression from the RIα/H9251 promoter and produced a 3.8-fold induction in FOXD2-expressing cells, indicating that the activating mechanism of PKB is enhanced by detachment of PKB from the membrane. Expressed as a control, the kinase-dead PKB mutant had no effect on the RIα/H9251 promoter. We next examined the effects of FOXD2 and/or PKBα on endogenous RIα protein levels at 16 and 24 h post-transfection by immunoblotting (Fig. 5D). We observed that FOXD2 up-regulated RIα protein levels up to 2-fold, whereas the combined expression of FOXD2 and PKBα elevated RIα-levels 2.7-fold.

**DISCUSSION**

We have previously shown that the forkhead transcription factor FOXC2 induces RIα expression in adipocytes (22) and that FOXD1 share the same function in testicular Sertoli cells (21). We report here the finding of FOXD2 mRNA expression in leukocytes and the regulation of RIα expression by FOXD2 in T cells. Spleen lymphocytes from FOXD2 null mutant mice had reduced levels of RIα mRNA and protein, whereas expression of
FIG. 6. Regulation of cAMP responsiveness by FOXD2 and PKBα sets threshold for modulation of T cell immune function.

Binding of a ligand to G-protein-coupled receptors (e.g. PGE2/EP-R) leads to the activation of adenyl cyclase, elevation of intracellular cAMP and thereby activation of PKA type I. PKA type I has been demonstrated to down-regulate mitogenic signaling through the TCR by activating Cbk/PAG-associated C-terminal Src kinase by phosphorylation on S384. C-terminal Src kinase inhibits Lck by phosphorylation of the C-terminal inhibitory site (Tyre)'. Activated Lck phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motifs leading to the recruitment of ZAP-70, which next is phosphorylated and activated by Lck. Activated ZAP-70 phosphorylates the adaptor protein called “linker for activation of T cells” (LAT). When phosphorylated, the linker for activation of T cells serves as a scaffold that couples downstream signaling pathways including phosphoinositide 3-kinase through interaction with its SH2 domain. PKB is recruited to the membrane via the phosphoinositide 3-kinase pathway and activated following stimulation of the TCR-CD3 complex and may travel to the nucleus. PKBα activity, released from the membrane, may increase the inductive effect of FOXD2 on the RIα promoter. This would thereby elevate the levels of the RIα subunit of PKA and tune PKA sensitivity and enhance the inhibitory effect of PKA on T cell signaling. The second messenger cAMP may also induce RIα expression in a FOXD2-dependent fashion and may thereby increase the sensitivity of its own effector system. Taken together, FOXD2 may together with PKBα and cAMP, induce PKA RIα expression and set the threshold for activation of PKA and negative modulation of T cell activation by cAMP.

both FOXD2 and FOXD1 induced the RIαb promoter as well as RIα protein in a Jurkat cell line. Furthermore, in the FOXD2-deficient mice, anti-CD3 activated spleen lymphocytes were less sensitive to inhibition of proliferation by cAMP than cells from wt mice as indicated by a right-shifted inhibition curve and elevated IC50 values. The observation that sensitivity to cAMP is reduced in FOXD2−/− mice may be due to the fact that (i) the levels of RIα are reduced and PKAIα (RIααCγ) has a 3–4-fold higher affinity and lower Kd for cAMP than the PKAIα (RIβαCγ) enzyme also present in T cells and (ii) PKAIα is the PKA isoform specifically recruited to the TCR complex (2). In support of this, we observed an up-regulation of RIα promoter activity and RIα protein in FOXD2-overexpressing cells. The RIαb promoter was induced 2–3-fold by cAMP in Jurkat T cells and about 5-fold in normal T cells (not shown). We also show here that overexpression of FOXD2 markedly increases the sensitivity of the RIαb promoter to cAMP in Jurkat cells, which may also involve endogenous FOXD2. A similar connection between FOXD1 and cAMP-mediated regulation of the RIαb promoter has been reported in Sertoli cells (21). We have previously also reported that translation of the RIαb mRNA may be stimulated by cAMP in testicular Sertoli cells depending on specific sequences in the RIαb 5‘-untranslated region (15). Furthermore, our findings showed that both cAMP and PKBα activate the RIαb promoter to a similar extent in the presence of FOXD2 in Jurkat T cells. However, in our assays the effects of PKBα and cAMP did not appear to be additive (not shown). A possible explanation for this may be that FOXD2 is a cross-road for PKA/PKBα cross-talk or that a mechanism of cAMP-mediated activation of PKBα is involved. Cyclic AMP has been demonstrated to stimulate PKB activity independently of PKA (27, 28), and phosphoinositide 3-kinase-independent activation of PKB by PKA has also been reported (29). Notably, Jurkat cells lack PTEN (phosphatase and tensin homology detected at chromosome 10) and have a disturbed phosphoinositide signaling (30).

The inductive effect of PKBα on FOXD2 in T cells and on FOXD1 in Sertoli cells is in contrast to the inhibitory effects reported on a different family of forkheads, the FOXO family. PKB/Akt is a key player in transduction of anti-apoptotic and proliferative signals in T cells, and overexpression of active PKBα/Akt1 promotes T cell survival (31). PKB is activated following proliferative signals through the interleukin-2 receptor and the T cell receptor complex in a phosphoinositide 3-kinase-dependent fashion (31, 32) and is negatively regulated by the tumor suppressor PTEN (30). PKB has the ability to antagonize apoptotic signals by inhibiting the function of winged helix transcription factors of the FOXO family members (AFX, FKHR, and FKHR-L1) via a direct phosphorylation and nuclear exclusion (reviewed in Ref. 20). FOXD2 and FOXD1 share little sequence similarity with FOXO proteins in regions outside the DNA-binding region, and we did not find consensus PKB phosphorylation sites. However, the possibility exists that FOXD2 competes for DNA-binding sites with FOXO proteins and that nuclear exclusion of the FOXO family members by PKB takes part in the synergistic effect of PKB and FOXD2. Based on the above, it is possible that PKB, in addition to its strong role in T cell activation, in parallel participates in activating immunomodulatory effects via the PKA pathway. In testicular Sertoli cells, we observed that ectopically expressed FOXD2 did not regulate the RIαb promoter regardless of the presence of PKBα (not shown), whereas FOXD1 and PKBα induced RIαb 15-fold in these cells (21). This led us to believe that additional cell-specific factors are essential for the different effects of the two FOXD proteins.

In conclusion, our data indicate that FOXD2 is a physiological regulator of RIα levels in T lymphocytes in vitro by inducing the RIαb promoter, working with PKB to further induce PKA RIα expression. Through this mechanism FOXD2 may regulate PKA RIα levels that regulate sensitivity to cAMP and set the threshold for negative modulation of T cell activation (Fig. 6).

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