Akt phosphorylates and suppresses the transactivation of retinoic acid receptor α

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The transactivation of nuclear receptors is regulated by both ligand binding and phosphorylation. We previously showed that RAR α (retinoic acid receptor α) phosphorylation by c-Jun N-terminal kinase contributes to retinoid resistance in a subset of NSCLC cells (non-small cell lung cancer cells), but the aetiology of this resistance in the remainder has not been fully elucidated [Srinivas, Juroske, Kalyankrishna, Cody, Price, Xu, Narayanan, Weigel and Kurie (2005) *Mol. Cell. Biol.* **25**, 1054–1069]. In the present study, we report that Akt, which is constitutively activated in NSCLC cells, phosphorylates RAR α and inhibits its transactivation. Biochemical and functional analyses showed that Akt interacts with RAR α and phosphorylates the Ser⁹⁶ residue of its DNA-binding

domain. Mutation of Ser⁹⁶ to alanine abrogated the suppressive effect of Akt. Overexpression of a dominant-negative form of Akt in an NSCLC cell line decreased RAR phosphorylation, increased RAR transactivation and enhanced the growth-inhibitory effects of an RAR ligand. The findings presented here show that Akt inhibits RAR transactivation and contributes to retinoid resistance in a subset of NSCLC cells.

Key words: Akt, non-small cell lung cancer cell (NSCLC cell), phosphorylation, retinoic acid receptor α (RAR α), retinoid resistance, transactivation.

INTRODUCTION

RARs [RA (retinoic acid) receptors] are ligand-dependent transcription factors that regulate genes involved in various biological processes, such as pattern formation, reproduction, cell differentiation and maintenance of tissue homeostasis [1,2]. RARs and RXRs (retinoid X receptors) bind to their target response elements as heterodimers and regulate gene expression in response to their ligands, all-*trans* RA and 9-*cis* RA [3]. In the absence of ligand, RXR–RAR heterodimers associate with a multiprotein complex containing transcriptional co-repressors that induce histone deacetylation, chromatin condensation and transcriptional suppression. Ligand binding causes the receptors to dissociate from the co-repressors and associate with co-activators that have histone acetyltransferase activity and induce local chromatin decondensation, recruitment of the RNA polymerase II holoenzyme and activation of target gene transcription [4,5].

Three major classes of co-activators have been studied in detail regarding their role in co-activating nuclear receptors. Members of the Swi/Snf/Brg class are involved in ATP-dependent remodelling of chromatin [6]. Members of the second class, which includes CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300, P/CAF (p300/CBP-associated factor) and p160/SRC (steroid receptor co-activator) have intrinsic histone acetyltransferase activity and have a crucial role in histone modification of transcriptionally active genes [7]. Members of the third

class belong to TRAP (thyroid hormone receptor-associated protein)/DRIP (vitamin D receptor-interacting protein)/ARC (activator-recruited cofactor) complexes, interact with ligand-bound nuclear receptors and stimulate transcription of *in vitro* DNA templates [8]. Co-activators contain one to several copies of leucine-rich LXXLL motifs in their receptor interaction domains. Crystallographic and biochemical studies have revealed that these LXXLL motifs interact with the ligand-activated AF-2 (activation function-2) domain of nuclear receptors, thereby providing a molecular basis for nuclear receptor-co-activator recruitment [9–11].

In addition to being activated by RA, RAR transactivation is regulated by phosphorylation. RARs are substrates for a variety of serine/threonine kinases, including PKA (protein kinase A), PKC, CDK7 (cyclin-dependent kinase 7) and p38 [12–16]. PKC phosphorylates RAR α and strongly reduces its transactivation by inhibiting its dimerization with RXR α [13], whereas CDK7 increases RAR transactivation by phosphorylating its A/B domain [14]. We have shown that activation of JNK (c-Jun N-terminal kinase) by stress signals leads to phosphorylation of RAR α , resulting in ubiquitin-mediated proteasomal degradation of the receptor [17]. Another serine/threonine kinase previously shown to phosphorylate nuclear receptors is PKB/Akt, a downstream mediator of class I PI3K (phosphoinositide 3-kinase) [18,19]. Akt phosphorylates the AF-1 domain of oestrogen receptor- α , thereby increasing its activity in a ligand-independent manner [20]. In contrast, Akt suppresses the transactivation of the androgen

Abbreviations used: AF, activation function; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; CDK7, cyclin-dependent kinase 7; DBD, DNA-binding domain; DR5, direct repeat separated by 5 nt; EMSA, electrophoretic mobility-shift assay; GSK3, glycogen synthase kinase 3; GST, glutathione S-transferase; HA, haemagglutinin; HEK-293T cells, human embryonic kidney 293T cells; IGF-I, insulin-like growth factor-I; JNK, c-Jun N-terminal kinase; LBD, ligand-binding domain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NSCLC cell, non-small cell lung cancer cell; p70^{S6K}, p70 S6 kinase; PARP, poly(ADP-ribose) polymerase; P/CAF, p300/CBP-associated factor; P-GSK3, phosphorylated GSK3; PPAR γ , peroxisome-proliferator-activated receptor γ ; PGC-1, PPAR γ co-activator-1; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PTEN, phosphatase and tensin homologue deleted from chromosome 10; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; RXR, retinoid X receptor; SRC, steroid receptor co-activator; TK, thymidine kinase; TTNPB, E-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid.

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receptor [21]. Beyond its role in regulating nuclear receptor function, Akt is the product of a proto-oncogene and phosphorylates a number of substrates that are important regulators of cell survival and transformation [22].

We previously showed that RAR α is aberrantly phosphorylated in lung cancer cells by JNK and that RAR α phosphorylation contributes to the blockade of retinoid signalling observed in a subset of lung cancers [17]. However, JNK is not responsible for the retinoid signalling defects observed in most of the retinoid-resistant NSCLC (non-small cell lung cancer) cell lines. Retinoids have demonstrated efficacy in the treatment of certain cancers but are ineffective in the treatment of lung cancer patients in part due to a defect in the transactivation properties of RARs in lung cancer cells, the aetiology of which has not been fully defined [23]. In the present study, we examined whether this retinoid resistance is instead mediated by Akt, which is constitutively active in NSCLC cell lines through amplification of genes encoding the p110 α catalytic subunit of PI3K (*PI3KCA*) and ErbB family members, epigenetic silencing of the *PTEN* gene and activating mutations in the genes *PI3KCA*, *KRAS* and *EGFR* [24–27]. We found that Akt phosphorylates RAR α at Ser⁹⁶ and inhibits its transactivation. We also found that inhibition of Akt enhances RAR activity and the growth-inhibitory effects of retinoids on NSCLC cells. Together, our findings support the hypothesis that Akt contributes to the retinoid resistance commonly found in NSCLC cells.

EXPERIMENTAL

Reagents

We purchased all-*trans* RA, TTNPB [*E*-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid], 9-*cis* RA and IGF-I (insulin-like growth factor-I) from Sigma-Aldrich (St. Louis, MO, U.S.A.); [γ -³²P]ATP, [³²P]P_i and [³²P]dCTP from ICN Biomedicals (Costa Mesa, CA, U.S.A.); antibodies against human RAR α and PARP [poly(ADP-ribose) polymerase] from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); polyclonal antibodies against Akt, Ser⁴⁷³-phosphorylated Akt (P-Akt), Ser²¹/Ser⁹-phosphorylated GSK3 (glycogen synthase kinase 3), GSK3, Thr³⁸⁹-phosphorylated p70^{S6K} (p70 S6 kinase), p70^{S6K} and PTEN (phosphatase and tensin homologue deleted from chromosome 10) from Cell Signaling Technology (Beverly, MA, U.S.A.); monoclonal antibodies against FLAG and tubulin from Sigma-Aldrich; anti-HA (haemagglutinin) antibody from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.); and purified recombinant phosphorylated and unphosphorylated Akt from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Cell culture

COS-1, HEK-293T (human embryonic kidney 293T), HeLa and NSCLC cells (H1299, Calu-1 and H460) were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). COS-1, HEK-293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37°C. NSCLC cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C.

cDNA constructs

HA-Akt, HA-Akt (T308D/S473D or DD mutant) and HA-Akt (K179A/T308A/S473A or AAA mutant) expression vectors were gifts from Dr Gordon Mills (M. D. Anderson Cancer Center). The Myr-Akt (where Myr stands for myristoylation) vector was constructed by inserting the myristoylation/palmitoylation signal of

Lck tyrosine kinase [28] into the N-terminus of Akt. The FLAG-RAR α , RAR β -luc, DR5TK-luc (where DR5 is direct repeat separated by 5 nt, and TK is thymidine kinase), TK-luc, GALTK-luc (where GAL is GAL4 DNA-binding sequence) and GST (glutathione S-transferase)-RAR α deletion constructs have been described elsewhere [17]. The GAL-RAR α (where GAL is yeast GAL4 protein) expression plasmids were constructed by cloning the cDNA encoding RAR α into the pFA-CMV vector (Stratagene, La Jolla, CA, U.S.A.). The S95A, S96A, S115A, S154A and S157A mutations were introduced into the GAL-RAR α by site-directed mutagenesis using the QuikChange[®] mutagenesis kit (Stratagene).

Purification of GST-tagged proteins

GST-tagged wild-type RAR α and deletion mutants were expressed and purified from the *BL21* bacterial strain (Stratagene) according to manufacturer's instructions. The integrity of the purified proteins was assayed by Coomassie Blue staining and Western blotting with anti-GST antibodies.

In vivo labelling and in vitro kinase assay

For the *in vivo* labelling experiments in HeLa cells, Lipofectamine[™] 2000 (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) was used to co-transfect HeLa cells (1.5×10^6 cells per 10 cm dish) with plasmids expressing HA-Akt or Myr-Akt and FLAG-tagged, wild-type RAR α . After 24 h, the cells were serum-starved overnight in phosphate-free medium and then incubated in the same medium for 4 h in the presence of 250 μ Ci/ml [³²P]P_i. Where indicated in the Figure legends, the cells were exposed to IGF-I (100 ng/ml) 1 h before harvesting. Cells were lysed in radio-immunoprecipitation assay buffer containing 100 nM okadaic acid, 1 mM orthovanadate and protease inhibitor cocktail (Sigma-Aldrich). FLAG-tagged RAR α was immunoprecipitated with anti-FLAG antibodies and the immunoprecipitates were subjected to electrophoresis and autoradiography. For *in vivo* labelling experiments in Calu-1 cells, cells were incubated with 250 μ Ci/ml [³²P]P_i for 8 h and RAR α was immunoprecipitated with anti-RAR α antibodies.

For the *in vitro* kinase assays, recombinant active Akt was incubated with 2 μ g of substrate proteins in kinase buffer containing 25 mM Hepes, 25 mM MgCl₂, 25 mM β -glycerophosphate, 20 μ M ATP, 0.5 mM dithiothreitol and 10 μ Ci of [γ -³²P]ATP for 30 min at 37°C. The labelled proteins were separated by SDS/PAGE and detected by autoradiography.

For stoichiometric analysis of phosphorylation, 0.75 μ g of purified GST-RAR α was incubated with increasing amounts of recombinant active Akt in kinase buffer containing 5 μ Ci of [γ -³²P]ATP for 30 min. The labelled proteins were separated by SDS/PAGE. A GST-RAR α band that exhibited saturated levels of phosphorylation was excised from the gel, and radioactivity in the protein was quantified by liquid-scintillation counting. The amount (mol) of phosphate incorporated per mol of GST-RAR α was calculated.

Phospho amino acid analysis

GST-RAR α DBD (DNA-binding domain) (5 μ g) was subjected to *in vitro* Akt kinase reaction and the labelled protein was separated by SDS/PAGE. The phosphorylated band corresponding to GST-RAR α DBD, visualized by autoradiography, was excised and phospho amino acid analysis was performed as described previously [29].

Luciferase assays

COS-1, HEK-293T and NSCLC cells (8×10^4 cells/well) were transfected in 24-well plates with the DR5TK-luc (200 ng), RAR β -luc (200 ng) or GALTK-luc (50 ng) reporter constructs, along with plasmids expressing Akt or GAL–RAR fusion proteins (50 ng). pRLTK-luc (50 ng) was transfected as an internal control. The total amount of plasmid was kept to 1 μ g/well. Cells were serum-starved overnight 24 h later and treated with all-*trans* RA or TTNPB (10^{-6} M) for 15 h, and cell lysates were assayed for luciferase activity using a dual-luciferase reporter assay system (Promega, Madison, WI, U.S.A.). Results were expressed as relative luciferase activity, the ratio of firefly to *Renilla* luciferase activity. Each experiment was performed three times, and results from a representative experiment are shown as the means $\pm$ S.D. (indicated by error bars) from three identical wells.

EMSA (electrophoretic mobility-shift assay)

DR5 oligonucleotides (5'-AGCTTAAGGGTTCACCGAAAG-TTCACTCGCAT-3') and an NF- κ B (nuclear factor κ B) binding site used as non-specific oligos (5'-TTGGGGGAAGGG-GGAATCTCTAGGCAAAGG-3') were used in the present study. Recombinant GST–RAR α (40 ng) and *in vitro*-translated RXR α were incubated with 32 P-labelled DR5 oligos for 30 min at 4°C in a binding buffer (10 mM Tris/HCl, 40 mM KCl, 6%, v/v, glycerol, 1 mM dithiothreitol and 0.05% Nonidet P40). Where indicated in the Figure legends, GST–RAR α was pre-incubated with active Akt kinase for 20 min in the presence of 1 mM ATP at 30°C. The RXR–RAR heterodimers bound to an RARE (RA response element) were resolved on 6% (w/v) polyacrylamide gels and subjected to autoradiography.

GST pull-down assays

GST and wild-type GST–RAR α (3 μ g) were immobilized on glutathione–agarose beads and incubated with purified Akt for 30 min on ice in binding buffer [buffer A (25 mM Tris/HCl, 150 mM NaCl and 0.1% Triton X-100)]. Protein complexes were washed twice with the same buffer, and bound materials were resolved by SDS/PAGE and subjected to Western blotting with anti-Akt antibodies.

Co-immunoprecipitation experiments

To study Akt–RAR interactions, COS-1 cells were transfected with plasmids expressing HA–Akt and FLAG–RAR α . After 48 h, the cells were lysed in buffer A, and the lysates were incubated with anti-FLAG or control IgG antibodies at 4°C. The immunoprecipitates were washed twice with buffer A and subjected to Western blotting with anti-HA antibodies.

Generation of stably transfected cell lines

H1299 and Calu-1 NSCLC cells were transfected with HA–Akt(DD) and HA–Akt(AAA) constructs respectively using LipofectamineTM (Invitrogen). Transfectants were selected in G418 (600 μ g/ml) for 2 weeks, and individual clones were isolated and later analysed for expression of transfected gene products.

Quantitative PCR

H1299 cells stably transfected with empty vector or Akt(DD) were treated with RA (10^{-6} M) for 5 h. The cells were harvested, and total RNA was extracted using an RNA isolation kit (Qiagen, Valencia, CA, U.S.A.). Reverse-transcriptase reactions were performed with 1 μ g of total cellular RNA using specific reverse

primers for RAR β and cyclophilin. The reverse-transcriptase reaction (10 μ l) was added to 40 μ l of PCR mixture containing 1 $\times$ PCR buffer (20 mM Tris, pH 8.4, and 50 mM KCl), 400 nM primers, 5 mM MgCl₂, ROX reference dye (Invitrogen), Taq polymerase and 100 nM fluorogenic probe labelled with 6-carboxy-fluorescein and a quencher. Amplification and quantification were based on real-time monitoring of probe degradation and were carried out using an ABI Prism 7700 performing 40 cycles at 94°C for 12 s and 60°C for 30 s. RAR β transcript levels were normalized to those of cyclophilin mRNA, which was used as a control.

Cell proliferation assays

Calu-1 cells (1×10^3) stably transfected with empty vector or Akt(AAA) construct were grown in RPMI 1640 medium containing 1% fetal bovine serum in 96-well plates. After 24 h, the cells were treated for 8 days with different concentrations of TTNPB as indicated in the Figure. Ligand was replenished every 48 h. Relative cell density was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay, as described elsewhere [30].

Statistical analysis

Statistical analyses were performed using one-way ANOVA test for luciferase assays and two-way ANOVA test for cell proliferation assays.

RESULTS

Akt represses ligand-dependent activation of retinoid receptors

We began investigating the effect of Akt on retinoid signalling with HEK-293T cells, because they express functional RAR and RXR proteins that transcriptionally activate RAREs in response to ligand [31]. We transiently transfected HEK-293T cells with a reporter construct containing the DR5 response element to which the RXR–RAR heterodimeric complex binds. Treatment with the RAR ligand all-*trans* RA led to a 5-fold increase in DR5TK-luc activity, whereas the activity of the control TK-luc reporter was unchanged (Figure 1A). Co-transfection of increasing amounts of Myr–Akt, an activated form of Akt [28], inhibited RA-induced DR5TK-luc activity in a dose-dependent manner (Figure 1A). To control for the possibility that PH domains suppressed RAR transactivation, we transfected HEK-293T cells with dominant-negative Akt (AAA mutant), which had no effect on DR5TK activity (Figure 1A).

We also co-transfected HEK-293T cells with Akt and a reporter construct driven by the *RARB* gene promoter, which contains the DR5 response element. Myr–Akt, but not Akt(AAA), suppressed RA-induced activation in those cells, suggesting that Akt represses the DR5 response element in the context of a natural promoter (Figure 1B). Stable transfection of H1299 NSCLC cells with Akt(DD), another constitutively active form of Akt, stimulated Akt-dependent signalling, as shown by increased expression of P-GSK3 (phosphorylated GSK3) (Figure 1C), and suppressed RA-induced *RARB* expression (Figure 1D). Together, these findings indicate that activation of Akt inhibits ligand-dependent activation of retinoid receptors.

Akt phosphorylates DBD of RAR α

We performed *in vivo* labelling experiments with [32 P]P_i to determine whether Akt activation increases RAR α phosphorylation in cells. RAR α phosphorylation increased in cells transfected with Myr–Akt (Figure 2A). To determine whether RAR α is a

Figure 1 Akt down-regulates ligand-dependent activation of RXR-RAR heterodimers

(A) Left panel: DR5TK-luc reporter activity in HEK-293T cells transiently co-transfected with increasing amounts of Myr-Akt or Akt(AAA). Right panel: control TK-luc activity in HEK-293T cells, with or without Myr-Akt. (B) RARβ-luc activity in HEK-293T cells, with or without Akt constructs. Relative luciferase activity in (A, B) is the ratio of firefly to *Renilla* luciferase activity. **P* < 0.01 (*n* = 3, one-way ANOVA test) for empty vector transfected versus Myr-Akt cells, in the presence of ligand. Expression levels of HA-tagged Akt constructs are shown by Western blotting with anti-HA antibodies. (C) Western-blot analysis of lysates of H1299 cells stably expressing empty vector (EV) or Akt(DD), using antibodies to P-GSK3 and GSK3. Results from two separate clones of Akt(DD) transfectants are illustrated. (D) Quantitative PCR analysis of RARβ mRNA levels in H1299 cells stably expressing empty vector (EV) or Akt(DD) construct (two clones).

substrate of Akt, we looked for evidence of physical interactions between the two. In COS-1 cells, HA-tagged Akt associated with FLAG-tagged RARα (Figure 2B). *In vitro* kinase assays showed that purified, active Akt phosphorylates GST-RARα (Figure 2C) and purified Akt kinase interacted with GST-RARα *in vitro* (Figure 2D). Stoichiometric analysis of the *in vitro* kinase reaction revealed that 33 % of the purified RARα protein was phosphorylated by Akt.

We next used deletion mutants to determine the regions of RARα that are phosphorylated by Akt. Akt phosphorylated the N-terminus (residues 1–187) and DBD, but not the LBD (ligand-binding domain), of RARα (Figure 2C). Phospho amino acid analysis of RARα DBD revealed phosphoserine residues, consistent with Ser/Thr-specific kinase activity of Akt (Figure 2E).

DBD of RARα is required for Akt-mediated repression

The inhibition of nuclear receptor function by phosphorylation may involve mechanisms that reduce DNA-binding activity, nuclear export, or stability of the receptor [32–34]. We investigated

the effect of Akt on the DNA-binding activity of RARs by performing EMSAs. Recombinant RARα was subjected to *in vitro* kinase reactions with Akt and then incubated with *in vitro*-translated RXRα and ³²P-labelled DR5 probe. We did not observe any change in the DNA-binding activity of the RXR-RAR heterodimer after the kinase reaction (Figure 3A).

To examine the effect of Akt activation on RARα protein stability and localization, we transiently transfected HEK-293T cells with Myr-Akt. Nuclear and cytosolic fractions of the cells were prepared, and RARα protein levels were assayed. Myr-Akt expression did not alter the protein levels in the nuclear fraction or increase the cytoplasmic localization of RARα (Figure 3B). PARP and α-tubulin were used as loading controls for nuclear and cytosolic fractions respectively. Taken together, these results suggest that the down-regulation of RARα function by Akt does not involve changes in DNA-binding activity, stability, or localization of RARα.

We next examined whether Akt would repress RARα transactivation when fused to a heterologous DBD. We transfected HEK-293T cells with a GAL4 DBD-RARα expression plasmid,

Figure 2 RAR α is a substrate of Akt

(A) Phosphorylation of RAR α in HeLa cells. Top panel: FLAG-RAR α was immunoprecipitated (IP) using anti-FLAG antibodies from lysates of cells transfected with empty vector (–) or Myr-Akt, and the immunoprecipitate was visualized by autoradiography. Bottom panel: FLAG-RAR α levels were measured by Western blotting (WB) with anti-FLAG antibodies. (B) Co-immunoprecipitation of FLAG-RAR α and HA-Akt in COS-1 cells. Upper panel: cell lysates were subjected to immunoprecipitation (IP) using mouse IgG or anti-FLAG antibodies followed by Western blotting with anti-HA antibodies. Lower panel: HA-Akt protein levels in cell extracts were measured by Western blotting with anti-HA antibodies. (C) *In vitro* kinase assay of Akt, using recombinant wild-type GST-RAR α or deletion mutants as substrates. Upper panel: phosphorylated proteins were resolved by SDS/PAGE followed by autoradiography. Phosphorylated bands of the appropriate molecular masses are indicated with arrows. A non-specific, 60 kDa band was seen in all lanes. Lower panel: gel loading was verified by Coomassie Blue staining. (D) Western-blot analysis of purified Akt protein incubated with GST or GST-RAR α immobilized on glutathione beads. (E) Phospho amino acid analysis of GST-RAR α DBD (82–167 amino acids) following *in vitro* kinase reaction with Akt.

along with a reporter construct containing a GAL4 response element (GALTK-luc). Because RXR is not required, we used TTNPB, a synthetic RAR ligand, to transactivate GAL-RAR. Expression of Myr-Akt substantially inhibited ligand-induced transactivation of GAL-RAR α but not GAL4 (Figure 3D). Myr-Akt had no effect on GAL-RXR α function (Figure 3E), suggesting that Akt represses retinoid signalling through RAR.

RAR α contains two activation domains (AF-1 and AF-2) that are required for maximal activation by its ligand. AF-1 and AF-2 are within the A/B region and LBD of RAR α respectively. We investigated which of these domains is required for the suppression of RAR α transactivation by Akt. We transiently co-transfected HEK-293T cells with Myr-Akt along with GAL-RAR Δ AB (which lacks the AF-1 domain) or GAL-RAR Δ AB/DBD (which lacks both DBD and AF-1 domain). In the absence of Myr-Akt, compared with wild-type RAR α , the transactivation of GAL-RAR Δ AB was decreased in the presence of ligand. This was expected because of the known synergy between the AF-1 and AF-2 domains [35,36]. The transactivation of GAL-RAR Δ AB was repressed by Myr-Akt, whereas the transactivation of the Δ AB/DBD RAR construct was resistant to down-regulation by Akt (Figure 3D), suggesting that the DBD is required for repression by Akt. To confirm this further, we transfected HEK-293T cells with GAL-RAR Δ DBD, a construct that lacks the DBD but retains both AF-1 and AF-2 domains. Myr-Akt failed to repress the transactivation of GAL-RAR Δ DBD (Figure 3D). To control for the possibility that PH domains suppressed GAL4 chimaeric protein transactivation, we transfected HEK-293T cells

with Akt(AAA), which had no effect on wild-type GAL-RAR α or GAL-RAR Δ DBD (Figure 3D).

Ser⁹⁶ is an Akt phosphorylation site

Sequence analysis of the DBD revealed no consensus Akt phosphorylation sites. We therefore created a series of GAL-RAR α constructs in which all the serine residues in this region were individually mutated to alanine residues, and we analysed the constructs for transactivation function. Myr-Akt suppressed ligand-induced transactivation of all GAL-RAR α mutant constructs except the S96A mutant (Figure 4A). In the *in vitro* kinase assays using RAR α DBD as the substrate, Akt phosphorylated wild-type GST-DBD but not the S96A mutant (Figure 4C), indicating that Ser⁹⁶ is an Akt phosphorylation site.

To investigate the functional significance of Ser⁹⁶ phosphorylation in the context of RXR-RAR heterodimers, we examined the ability of the S96A mutation to block Akt-mediated repression of DR5 transactivation by RXR-RAR heterodimers. We observed that, in the context of a DR5 element, ligand-induced transactivation of RAR α S96A mutant was less than that of wild-type RAR α (Figure 4D), which was not the case in the setting of GAL chimaeric constructs (Figure 4A). Furthermore, Myr-Akt repressed ligand-induced DR5 transactivation in COS-1 cells co-transfected with wild-type RAR α but not those co-transfected with RAR α S96A (Figure 4D). EMSA revealed that RAR α S96A bound to a DR5 element and heterodimerized with RXR α as well as

Figure 3 The DBD of RAR α is required for Akt-mediated repression

(A) Analysis of DNA-binding activity of RXR–RAR heterodimers after GST–RAR α was subjected to *in vitro* kinase reactions, with or without active Akt (P-Akt). DNA–protein complexes were resolved by SDS/PAGE and autoradiography. (B) Western blots of HEK-293T cells transiently transfected with empty vector (–) or Myr–Akt construct. Cells were lysed 48 h after transfection, and nuclear (N) and cytosolic (C) fractions were analysed for the indicated proteins. PARP and tubulin were used as loading controls for the nuclear and cytosolic fractions respectively. (C) Schematic representation of wild-type and mutant GAL–RAR α constructs. (D) Effect of Myr–Akt or Akt(AAA) co-transfection on transactivation of wild-type (WT) and mutant GAL–RAR α constructs. Relative luciferase activity is the ratio of firefly to *Renilla* luciferase activity. * $P < 0.01$ ($n = 3$, one-way ANOVA test) for empty vector-transfected versus Myr–Akt cells, in the presence of ligand. (E) Effect of Myr–Akt co-transfection on transactivation of GAL–RXR α . Relative luciferase activity is the ratio of firefly to *Renilla* luciferase activity. (F) Expression levels of GAL–RAR constructs are shown by Western blotting with anti-GAL4 antibodies. Bands of expected molecular masses are indicated with arrows. Lower-molecular-mass band in each lane may represent a truncated form of GAL chimaeras.

wild-type RAR α did (Figure 4E), indicating that the reduced DR5 transactivation by RAR α S96A was not due to impaired DNA-binding or heterodimerization properties. Sequence analysis of RAR β and RAR γ revealed that Ser⁹⁶ is a conserved residue (Figure 4F). We therefore postulated that Akt also inhibits these RAR subtypes. Indeed, Myr–Akt, but not Akt(AAA), repressed transactivation of GAL–RAR β in HEK-293T cells (Figure 4G). Together, these findings indicate that RAR Ser⁹⁶ phosphorylation is required for the suppression of RXR–RAR transactivation by Akt.

Akt contributes to retinoid resistance in NSCLC cells

Although many NSCLC cells express normal levels of retinoid receptors, they are not sensitive to the biological effects of RA in part because of a defect in RAR transactivation function [23]. To test our hypothesis that high Akt activity contributes to RAR dysfunction in NSCLC cells, we inhibited Akt signalling in two NSCLC cell lines, Calu-1 and H460, which differed with respect to basal Akt activation, as shown by Western blotting of P-Akt (Figure 5A). Akt is constitutively phosphorylated in Calu-1 cells in part due to its low expression of the PTEN lipid phosphatase

[37]. Inhibition of Akt by transient transfection of the dominant-negative mutant Akt(AAA) increased DR5TK activity in Calu-1 cells but not in H460 cells (Figure 5B), supporting a role for Akt in the suppression of RAR function in Calu-1 cells.

Finally, we examined whether the high Akt activity in Calu-1 cells contributes to the resistance of those cells to growth inhibition by the RAR ligand. Calu-1 cells stably transfected with Akt(AAA) displayed a block in Akt signalling, as shown by Western blotting for P-p70^{S6K} (Figure 5C). Metabolic labelling studies revealed that RAR α was phosphorylated to a lesser extent in Calu-1 Akt(AAA) transfectants than controls (empty vector transfectants) (Figure 5D). Compared with its effect on controls, TTNPB more profoundly inhibited the proliferation of Akt(AAA) transfectants (Figure 5E). Together, these findings support the hypothesis that Akt contributes to retinoid resistance in a subset of NSCLC cells.

DISCUSSION

In the present study, we explored the role of Akt in the regulation of RAR function and found that Akt phosphorylates RAR α and suppresses its transactivation. We identified an Akt

Figure 4 Akt phosphorylates Ser⁹⁶ of RAR α

(A) GALTK-luc activity in HEK-293T cells transiently co-transfected with equal amounts of wild-type (WT) GAL-RAR α or various mutants, in the presence or absence of the Myr-Akt or Akt(AAA) expression plasmid. (B) Expression levels of mutant RAR α constructs are shown by Western blotting using anti-GAL4 antibodies. Bands of expected molecular masses are indicated with an arrow. (C) *In vitro* kinase assays of Akt, using GST-tagged RAR α DBD and DBD S96A as substrates. Upper panel: phosphorylated proteins were resolved by SDS/PAGE followed by autoradiography. Lower panel: gel loading was verified by Coomassie Blue staining. (D) DR5TK-luc activity in COS-1 cells transiently co-transfected with wild-type RAR α or RAR α S96A, with or without Myr-Akt. Expression levels of FLAG-tagged wild-type and mutant RAR α proteins are shown by Western blotting using anti-FLAG antibody. (E) EMSA to analyse DR5 DNA-binding activity of RAR wild-type and S96A mutant proteins in the presence of RXR. *In vitro*-translated proteins were incubated with radioactive DR5 probe in the presence or absence of competitor, including 10-fold excess specific or non-specific non-radioactive oligonucleotides as shown in the Figure. (F) Sequence alignment of the conserved Ser⁹⁶ region of mouse (m), rat (r) and human (h) RAR α , and human RAR β and RAR γ subtypes. Ser⁹⁶ of hRAR α and corresponding serine residue of other members are shown in boldface. (G) GALTK-luc activity in HEK-293T cells transiently co-transfected with human GAL-RAR α , with or without Myr-Akt or Akt(AAA). Relative luciferase activity in (A, D, G) is the ratio of firefly to *Renilla* luciferase activity.

phosphorylation site on Ser⁹⁶ within the DBD of RAR α . Furthermore, Akt inhibition enhanced retinoid sensitivity in an NSCLC cell line. These findings indicate that RAR is a novel Akt substrate, that Akt is important in the regulation of RAR function, and that constitutive Akt activation may contribute to retinoid resistance in a subset of NSCLC cells.

Several lines of evidence suggest that RAR α is a direct substrate of Akt. We showed that RAR α physically associates with Akt *in vitro* (Figure 2D) and *in vivo* (Figure 2B). Akt also increased the phosphorylation of RAR α *in vitro* (Figure 2C) and *in vivo* (Figure 2A). Mutation of Ser⁹⁶ of the DBD to an alanine residue prevented Akt-mediated phosphorylation (Figure 4C) and restored transactivation (Figure 4A). Finally, Ser⁹⁶ is conserved

across species and RAR subtypes (Figure 4F). These results suggest that Ser⁹⁶ is a *bona fide* Akt phosphorylation site. However, Ser⁹⁶ does not resemble the consensus Akt phosphorylation site (RXRXXS/T) [38], and we do not know the mechanism by which Akt recognizes this region. There are other examples of Akt substrates that do not have the consensus motif, such as CREB and YB-1 (Y-box binding protein-1) [39,40]. Furthermore, although mitogen-activated protein kinases usually phosphorylate serine or threonine residues that are followed by proline, there are several physiological substrates such as TAB1 [TAK-1 (transforming growth factor- β -activated kinase-1) binding protein], cdc25 and microtubule-associated protein Tau where this is not observed [41–43].

Figure 5 Akt contributes to retinoid resistance in NSCLC cells

(A) Western-blot analysis of lysates of Calu-1 and H460 cells, using antibodies to P-Akt, Akt and PTEN. (B) DR5TK-luc activity in Calu-1 and H460 cells, in the presence or absence of the dominant-negative Akt(AAA) expression construct. Relative luciferase activity is the ratio of firefly to *Renilla* luciferase activity. (C) Western-blot analysis of lysates of Calu-1 cells stably expressing empty vector (EV) or Akt(AAA) construct, using antibodies to P-p70^{S6K} and p70^{S6K}. Results are illustrative of two separate Akt(AAA) transfectants. (D) Phosphorylation of RAR α in Calu-1 cells. Upper panel: after metabolic labelling, FLAG-RAR α was immunoprecipitated from cell lysates with anti-RAR α antibodies, and the immunoprecipitate was visualized by autoradiography. Lower panel: RAR α levels were measured by Western blotting with anti-RAR α antibodies. (E) MTT assay of Calu-1 cells stably expressing empty vector or Akt(AAA), in the absence or presence of different concentrations of TTNPB. Results are illustrative of two separate Akt(AAA) transfectants. Values are expressed as means $\pm$ S.D. from five identical wells and statistical analysis was performed by two-way ANOVA test.

We showed that the DBD of RAR α is important for mediating repression by Akt (Figure 3D), indicating that the DBD has functions other than DNA binding. Although nuclear receptors usually interact with co-regulatory proteins through their LBDs, there are several reports of DBDs being used as docking sites for co-activator recruitment. For example, Jun dimerization protein-2 interacts with the DBD of the progesterone receptor and stimulates AF-1 function by recruiting other co-activators such as CBP and P/CAF [44]. Similarly, the PPAR γ (peroxisome-proliferator-activated receptor γ) co-activator PGC-1 (PPAR γ co-activator-1) interacts with the DBD of PPAR γ . This interaction induces a conformational change in PGC-1 that enables its recruitment of other co-activators, such as SRC-1 and CBP/p300 [45]. Along similar lines, P/CAF has also been shown to interact with the DBDs of RXR and RAR *in vitro*. This RAR-P/CAF interaction potentiates the ligand-dependent transactivation of RA-responsive reporter constructs [46,47]. Thus we have identified a novel role of the DBD in RAR transactivation. While the mechanism has not been fully defined, one possibility is that RAR α phosphorylation by Akt inhibits the ability of RAR to recruit essential co-activators to the receptor complex.

Whereas normal human bronchial epithelial cells are sensitive to the growth-inhibitory effects of retinoids, NSCLC cells are typically resistant. Multiple mechanisms contribute to retinoid resistance in NSCLC cells. First, expression of *RARB*, which has tumour-suppressing properties [48,49], is silenced in certain NSCLC cell lines through DNA methylation and aberrant histone acetylation on the gene's promoter [50,51]. Secondly, we previously showed that RAR phosphorylation by JNK decreases RAR levels by inhibiting RAR stability in H322 NSCLC cells [17]. Thirdly, certain NSCLC cell lines are resistant to retinoids despite having high levels of retinoid receptors, but the receptors in those cells have transactivation defects [23]. Fourthly, we previously showed that certain retinoid-resistant NSCLC cells, including the Calu-1 cells studied in the present study, express co-activators at levels similar to those in retinoid-sensitive cells, suggesting that aberrant expression of co-activators does not have a role in retinoid resistance [51]. In the present study, we show that co-activator function may be aberrant in NSCLC cells with high Akt activity, thereby contributing to retinoid resistance.

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