Down-Regulation of the Tumor Suppressor Gene Retinoic Acid Receptor β2 through the Phosphoinositide 3-Kinase/Akt Signaling Pathway

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The retinoic acid receptor β2 (RARβ2) is a potent, retinoid-inducible tumor suppressor gene, which is a critical molecular relay for retinoid actions in cells. Its down-regulation, or loss of expression, leads to resistance of cancer cells to retinoid treatment. Up to now, no primary mechanism underlying the repression of the RARβ2 gene expression, hence affecting cellular retinoid sensitivity, has been identified. Here, we demonstrate that the phosphoinositide 3-kinase/Akt signaling pathway affects cellular retinoid sensitivity, by regulating corepressor recruitment to the RARβ2 promoter. Through direct phosphorylation of the corepressor silencing mediator for retinoic and thyroid hormone receptors (SMRT), Akt stabilized RAR/SMRT interaction, leading to an increased tethering of SMRT to the RARβ2 promoter, decreased histone acetylation, down-regulation of the RARβ2 expression, and impaired cellular differentiation in response to retinoid. The phosphoinositide 3-kinase/Akt signaling pathway, an important modulator of cellular survival, has thus a direct impact on cellular retinoid sensitivity, and its deregulation may be the triggering event in retinoid resistance of cancer cells. (Molecular Endocrinology 20: 2109–2121, 2006)
thereby tethering histone acetyl transferase activity to the promoter (15, 16).

Epithelial cancers are frequently associated with hyperactivation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. In response to growth factors, PI3K catalyzes the production of the lipidic second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) from PIP2 at the inner side of the cytoplasmic membrane. PIP3 contributes to the recruitment and activation of several downstream targets, including the serine/threonine kinase Akt (17). The PI3K/Akt pathway is a key regulator of cell survival through multiple downstream targets. This includes the inactivation of the proapoptotic factors Bad and procaspase-9 by phosphorylation, increased transcription of nuclear factor-κB and cAMP response element-binding protein survival genes or nuclear exclusion of the cyclin inhibitors p21 and p27 (18). Activation of PI3K/Akt signaling pathway can occur through several mechanisms. Amplification of the PI3K gene has been detected in ovarian and cervix cancers (19, 20) and also in several forms of epithelial cancers (21–23). Another mechanism involved in PI3K/Akt activation is the loss of the tumor suppressor phosphatase and tensin homolog (PTEN). PTEN is a 3‘ phosphatase that converts PIP3 back to PIP2, thus exerting a negative control on the PI3K signaling pathway. The loss of PTEN function by mutations is extremely common in prostate, breast, and lung cancers (24).

We previously investigated the mode of transcriptional regulation of the RARβ2 promoter in the retinoid-sensitive P19 embryonal carcinoma cell line. We showed that its regulation follows rules that differ significantly from the consensual model. First, the acetylation state of histones H3 and H4 associated to this promoter does not vary upon ligand treatment (25). We further demonstrated that the ligand-induced transcriptional activation of this promoter is correlated to histone H3 hyperphosphorylation and prevented by a retinoid antagonist, thereby establishing a relationship between the activation state of RAR and the phosphorylation state of histone H3 (26). In the experiments described here, we extend our studies of the mechanism underlying histone constitutive acetylation at the RARβ2 gene and its implication in transcriptional regulation. We demonstrate that activation of the PI3K/Akt signaling pathway induces transcriptional repression of the RARβ2 gene. This Akt-mediated repression is characterized by an enhanced recruitment of SMRT to the RARβ2 promoter, leading to histones H3 and H4 deacetylation and impaired biological responses to atRA. Akt-mediated phosphorylation of SMRT led to a strengthened interaction with RAR, thereby identifying the PI3K/Akt pathway as a major negative regulator of the tumor suppressor gene RARβ2 expression.

RESULTS

Histones Posttranslational Modifications and RNA Polymerase II (RNAP) Loading at the RARβ2 Promoter in P19 and HeLa Cells

To identify the epigenetic mechanism regulating RARβ2 expression, we first investigated the histone acetylation status in the retinoid responsive cell lines P19 and in a poorly retinoid responsive cell line, HeLa cells (27–29). As shown in Fig. 1A, P19 cells exhibited a rapid accumulation of the RARβ2 transcripts after atRA exposure, which was detectable after 30 min, whereas a delayed response was observed in HeLa cells (1 h). We also observed a considerable basal expression of this gene in P19 cells. In addition, RARβ2 transcripts reached a plateau after 4 h in HeLa cells instead of P19 cells, in which they continually increased even after 8-h treatment.

Antibodies specific for nonacetylated/nonphosphorylated or acetylated histone H3 were used to immunoprecipitate formaldehyde-cross-linked, sonicated chromatin from P19 and HeLa cells. A semiquantitative PCR analysis of input DNA and of immunoprecipitated DNA, for which conditions were adjusted to ensure linear amplification of DNA targets, was carried out to detect a fragment of the RARβ2 promoter encompassing several cis-acting elements, including the canonical DR5 (Fig. 1B). These chromatin immunoprecipitation (ChIP) analyses demonstrated a high level of acetylated histone H3 at the RARβ2 promoter with barely any detectable form of nonacetylated/nonphosphorylated H3 in the retinoid-responsive P19 cells (Fig. 1C). In contrast, only the nonacetylated/nonphosphorylated histone H3 was detected in HeLa cells at the same locus, showing that nonacetylated histone H3 is associated to a poorly transcriptionally active promoter.

Histone H3 K4 methylation is associated with active chromatin, whereas histone K9 methylation is associated with repressed chromatin (30). We thus investigated the level of methylation on K4 and K9 at the RARβ2 promoter in P19 and HeLa cells. Only trimethylated K4 was detected in P19 cells at the RARβ2 promoter, a posttranslational modification that was not further altered by atRA treatment. A similar analysis showed that none of these posttranslational modifications of histone H3 were detectable in HeLa cells, as well as histone H3 serine 10 phosphorylation, a hallmark of RARβ2 promoter activation in P19 cells (26).

We next assessed whether atRA treatment correlates with alteration of histone acetylation at the RARβ2 promoter. Constitutive acetylation of H3 and H4 was detected in the absence of ligand in retinoid-responsive P19 cells (Fig. 1E). Adding 100 nM atRA caused no further detectable changes after a 2-h treatment. Similar results were obtained for shorter or longer incubation times (25). In contrast, ChIP analysis revealed that ligand treatment of HeLa cells caused an
increase in the acetylation of H3 (4.2-fold induction) and of H4 (1.6-fold induction).

We previously demonstrated that RNAP was constitutively loaded at the RARβ2 promoter (Fig. 1F) (25). In sharp contrast, ChiP analysis in HeLa cells revealed that no RNAP could be detected at the nonstimulated RARβ2 promoter, and that a clear increase of RNAP loading occurred in response to atRA. These results thus show that the retinoid-responsive cell line P19 exhibits a high level of H3 and H4 acetylation at the RARβ2 promoter in absence of ligand and that this correlates with a constitutive RNAP loading. Because histone deacetylation at nuclear receptor-regulated promoters relies on corepressor recruitment and associated HDAC activities, we hypothesized that a specific regulation of corepressor complexes might occur in the retinoid-responsive P19 cells.

**RARβ2 Promoter H3 and H4 Hyperacetylation Is Linked to a Lack of Corepressor Binding to RAR/RXR Heterodimers**

Several studies indicated that corepressor activity is regulated through several mechanisms, including proteosomal degradation, nuclear exclusion, regulation of its association with others components of the corepressor complex, or their association with nuclear receptor themselves (31).
To unravel the molecular basis for the observed constitutive histone H3 and H4 acetylation, we first compared the level of expression of SMRT in P19 and HeLa cells by Western blot analysis. As shown in Fig. 2A, SMRT was detected in these cell lines in equal amounts and localized mostly to the nucleus, ruling out a regulation via nuclear exclusion. Nuclear localization of SMRT was also confirmed by immunocytochemistry, and similar results were obtained for NCoR (data not shown). The SMRT polypeptide displays a much higher affinity for RAR compared with NCoR (32) (Fig. 2, C and D). We therefore tested the functionality of SMRT in P19 cells using the one-hybrid system. As shown in Fig. 2B, transfection of increasing amounts of a Gal4DBD-SMRT expression vector led to a strong inhibition of the basal level of expression of a Gal4-tk luc promoter (>80% at 50 ng).

Taken together, these data demonstrate that corepressor complexes are expressed and functional in P19 cells. They further suggest that H3 and H4 constitutive acetylation on the RARβ2 promoter may be linked to a specific regulation of corepressor-RAR interaction. To test this hypothesis, we used a modified mammalian two-hybrid assay. A chimeric reporter gene, containing the −124/+14 fragment of the RARβ2 promoter driving the luciferase gene (RARβ2-luc), was cotransfected in HeLa or P19 cells either with an expression plasmid coding for VP16-AD (activation domain) or a VP16-AD fused to the C-terminal domain (including the nuclear receptor interacting domain (RID)) of NCoR or SMRT (Fig. 2C). In HeLa cells, only a marginal activation was observed upon cotransfection of VP16-NCoR (2-fold induction), whereas VP16-SMRT induced a strong activation (10-fold induction).
of the reporter system. However, when the same experiment was repeated in P19 cells, no activation could be detected, neither in the presence of VP16-SMRT nor of VP16-NCoR. This suggests that corepressor association to RAR may be defective. Because the RARβ2 promoter contains binding sites for several transcription factors (see Fig. 1B), we next wanted to establish whether this interaction was dependent solely on the presence of RAR/RXR heterodimer on the promoter. We thus performed a similar two-hybrid assay using a synthetic reporter gene bearing a trimerized RARE hooked to the tk promoter [(DR5)3-tk luc]. As previously shown for the RARβ2 promoter, a potent activation (65-fold increase) was observed in HeLa cells when this reporter gene was cotransfected with the VP16-SMRT fusion protein. Again, this activation was much weaker (<10-fold increase) in the presence of VP16-NCoR (Fig. 2D). No activation was detected in P19 cells. In addition, mutation in the proximal RARE of the RARβ2 promoter completely abolished the reporter gene activity in HeLa cells, demonstrating that the SMRT effect is only carried through RAR/RXR heterodimers (Fig. 2E). We therefore wanted to extend these results to the endogenous RARβ2 promoter using ChIP assay. In this experiment, we used antibodies directed against RARα, expressed predominantly in P19 and HeLa cells (28, 29, 33), and SMRT to immunoprecipitate formaldehyde-cross-linked, sonicated chromatin from untreated HeLa and P19 cells. As shown in Fig. 2F, RARα could be cross-linked to the RARβ2 promoter in HeLa and P19 cells. However, SMRT could be detected only in HeLa cells, thus establishing a direct correlation between the histone acetylation status and SMRT binding at the RARβ2 promoter.

Taken together, these results demonstrate that 1) the RXR/RAR heterodimer interacts preferentially with SMRT and 2) this interaction is regulated in a cell-specific manner (P19 vs. HeLa cells).

Akt Signaling Pathway Regulates the Interaction of SMRT with RAR/RXR

Several signaling pathways have been shown to regulate SMRT/nuclear receptor interactions. In particular, MEK-1 and MEK-1 kinase (MEKK-1) kinases can inhibit this interaction (34). However, overexpressing dominant-negative mutants of these kinases had no effect on RXR/RAR-SMRT interaction in P19 cells (data not shown). To identify signaling pathway(s) that could regulate this interaction, we screened a large panel of protein kinases inhibitors for their ability to alter the RXR/RAR-SMRT interaction in HeLa cells. Among them, we found that the PI3K kinase inhibitor LY294002 led to a strong decrease (>70% at 50 μM) of the reporter activity (Fig. 3A). We next wanted to know whether this signaling pathway was effective in regulating RXR/RAR-SMRT interaction in P19 cells. We used again our modified two-hybrid system, using the [(DR5)3-tk luc] reporter gene, in which the fusion pro-tein VP16-SMRT is overexpressed, together with either a constitutive active form of Akt1 (Akt-CA) or a dominant-negative form (Akt-dN). As shown in Fig. 3B, increasing concentrations of Akt-CA strongly stimulated the luciferase activity, whereas no effect was found upon overexpression of Akt-dN. Similar results were found with the RARβ2-luc reporter gene (data not shown). In contrast, expressing increasing amounts of Akt-CA in HeLa cells had no effect, whereas Akt-dN efficiently repressed the luciferase activity.
response (supplemental Fig. 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org).

An increased cellular Akt activity is thus correlated to a strong interaction between DNA-bound RXR/RAR heterodimer and SMRT. From this, it can be inferred that a strong RXR/RAR-SMRT interaction in HeLa cells results from a higher Akt activity in this cell line. To test this hypothesis, we evaluated the level of activity of this kinase in P19 and HeLa cells by an immunoprecipitation/kinase assay using glycogen synthase kinase 3 (GSK3) as a substrate. As shown in Fig. 3C, Akt activity in HeLa cells was at least four times higher than that detected in P19 cells, demonstrating that SMRT binding efficiency to RAR/RXR heterodimer is strongly correlated to cellular Akt activity.

SMRT Is a Direct Substrate for Akt Kinase

To determine how Akt could regulate RXR/RAR-SMRT interaction, we first analyzed whether a component of the RXR/RAR-SMRT complex was a direct substrate for Akt. Because RXR displays only a very low binding affinity for SMRT, RAR represented a possible target in the RXR/RAR heterodimer. We thus tested the interaction between an immobilized glutathione S-transferase (GST)-RAR fusion protein and radiolabeled Akt. No stable interaction could be detected in this system (data not shown). We next tested the ability of Akt to interact with SMRT R1D (amino acids 1028 to 1495). As shown in Fig. 4A, a specific interaction could be detected between SMRT R1D and Akt.

We then performed in vitro phosphorylation reactions using purified components (Fig. 4B). Incubation of the activated form of Akt with radiolabeled ATP led to Akt autophosphorylation (Fig. 4B, lane 1), whereas GSK3 was efficiently phosphorylated by this kinase (Fig. 4B, lane 2). Purified GST was not phosphorylated in these experimental conditions (Fig. 4B, lane 3). Incubating increasing amounts of activated Akt with purified GST-SMRT R1D led to phosphorylation of GST-SMRT, demonstrating that the SMRT R1D moiety is a substrate for Akt (Fig. 4B, lanes 4–7).

To assess the specificity of the reaction, we also performed an in vitro phosphorylation reaction using another component of the PI3K/Akt signaling pathway, the GSK3. As shown previously, the GST-SMRT fusion protein was phosphorylated in vitro by Akt kinase (Fig. 4C). This phosphorylation was specific because active GSK3 was unable to modify SMRT R1D.

Thus, Akt is able to interact with and to phosphorylate SMRT R1D, suggesting a possible interference with the RAR-SMRT interaction.

Identification of an Akt Phosphorylation Site in SMRT R1D

Analysis of the SMRT amino acid sequence using an algorithm at low stringency identified four potential phosphorylation sites for Akt (35), with three of them being located within SMRT R1D (T1112, S1407, S1434) (Fig. 5A). We thus introduced single alanine substitutions in the VP16-SMRT fusion protein, generating VP16-SMRT T933A, VP16-SMRT T1112A, VP16-SMRT S1407A, and VP16-SMRT S1434A mutants. Their activities were assayed in the modified two-hybrid assay in HeLa cells using the [(DR5)3-tk luc] reporter gene. As shown in Fig. 5B, the T933A, S1407A, and S1434A mutations did not impact on the luciferase response, whereas the T1112A mutation led to a 55% decrease of the reporter activity. We showed above that overexpression of a constitutively active mutant of Akt (Akt-CA) promoted the interaction between SMRT and RXR/RAR heterodimer in P19 cells. In this assay, the T933A, S1407A, and S1434A mutants were sensitive to Akt-CA overexpression,
whereas T1112A turned out to be insensitive, clearly indicating that T1112 mediates Akt effect on the SMRT-RXR/RAR interaction (Fig. 5C). This effect was specific because the T1112A mutation did not impede on the ability of SMRT to bind RAR in vitro (supplemental Fig. 2, published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org). Similar results were obtained using the RARβ2-luc reporter gene (data not shown).

T1112 is therefore very likely to be a major Akt phosphorylation site. We thus tested the ability of SMRT T1112A mutant to be phosphorylated by purified Akt kinase in vitro. As shown previously, the GST-SMRT fusion protein was phosphorylated in the presence of the activated form of Akt (Fig. 5D), whereas the T1112A mutation severely compromised Akt-catalyzed phosphorylation of SMRT RID (3.5-fold decrease).

Effect of Akt Activation on RARβ2 Promoter Activity and Retinoid Signaling in P19 Cells

To gain further insights in the role of Akt on the RARβ2 promoter activity, we established P19 subclones expressing Akt-CA. Two clones exhibiting distinct levels of Akt-CA expression were selected and characterized (Fig. 6A). As shown in Fig. 6B, the two clones displayed a reduced basal expression level of the RARβ2 gene when compared with wt P19 (see insert). Kinetic experiments at a saturating concentration of atRA (100 nM) showed lower amounts of RARβ2 transcripts at all time points for each Akt-CA subclones (>2-fold at 8 h). More importantly, RARβ2 transcripts reached a plateau after 4 h, in sharp contrast to wild-type cells, for which the level of RARβ2 transcripts continually increased even after 8 h of treatment. In addition, we also observed clearly reduced retinoid response at subsaturating concentration (1 and 10 nM) after a 20-h treatment (supplemental Fig. 3, published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org).

These data show that Akt activation impairs on RARβ2 promoter activity in response to retinoic acid.

We then aimed at establishing the ligand-independent recruitment of SMRT on the RARβ2 promoter in these two subclones using a ChIP assay. Western blot analysis revealed that similar amounts of SMRT were expressed in the two subclones compared with wt P19 (data not shown). As shown previously, no association
SMRT Regulation by Akt

Fig. 6. Effect of Akt Hyperactivation on the RARβ2 Promoter Activity in P19 Cells
A, Western blot analysis of the Akt-CA content in stable transfected P19 cells. A total of 50 μg of whole-cell extracts was analyzed as described in Materials and Methods using a monoclonal antibody against the HA tag. B, Quantification of RARβ2 promoter activity in wt P19, clone 1, and clone 2. Cells were treated with atRA (100 nM) for the indicated time, and RARβ2 transcripts were quantified by real-time PCR. Inset represents the basal level of wt P19, clone 1, and clone 2. All experiments were carried out in duplicate.

of SMRT to the RARβ2 promoter could be detected in wt P19 cells (Fig. 7A). However, SMRT could be cross-linked to the RARβ2 promoter in the absence, but not in the presence of 100 nM atRA in the two subclones. This observation provides direct evidence that SMRT recruitment on the RARβ2 promoter is dependent on the level of Akt activation in P19 cells. Paralleling the recruitment of SMRT, ChIP analysis using antibodies specific for the nonacetylated/nonphosphorylated or for acetylated histone H3 demonstrated that the amount of nonmodified H3 increased significantly upon expression of Akt-CA (Fig. 7B). We next assessed the effect of atRA treatment on H3 and H4 acetylation status at the RARβ2 promoter. We noticed that virtually no H4 acetylation could be detected in the two Akt-CA-expressing clones in the absence of ligand (Fig. 7C). Retinoid treatment led to an increase in the level of acetylation of histones H3 and H4 associated with this promoter (2.2- and 2-fold for H3; 3.3- and 2.8-fold for H4), in sharp contrast with wt P19 cells (Fig. 7C). As a consequence, a lower amount of RNApol was associated to the RARβ2 promoter (2.2- and 2.6-fold increase) (Fig. 7D). We next examined whether SMRT recruitment impacted on transcriptional elongation by studying histone acetylation levels and density of RNApol on the exon 3 of the RARβ2 gene. ChiP analysis revealed a low level of acetylation of H3 and H4 in the absence of ligand. AtRA treatment led to a strong increase in both H3 and H4 acetylation levels in wt P19 and in the two P19 Akt-CA subclones (supplemental Fig. 4, published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org). Low amounts of RNApol were detected on exon 3 in nonstimulated cells, and ligand addition strongly increased RNApol density on exon 3 in each cellular background.

We conclude from these experiments that Akt, by regulating the interaction of SMRT with RAR/RXR heterodimers, controls histones H3 and H4 acetylation level and RNApol loading at the RARβ2 promoter without affecting significantly transcriptional elongation.

P19 cells differentiate into neuronal cells upon retinoic acid treatment during cellular aggregation and the differentiation state is characterized by the expression of the neuron-specific marker βIII-tubulin. A dose-dependent increase of the expression of this marker was detected in P19 cells after atRA treatment (Fig. 8). Although the expression of βIII-tubulin increased after retinoid treatment in the two P19 Akt-CA clones, the maximal level of expression was lower than that observed in wt P19 cells (2.8- and 2-fold vs. 4.2-fold).

Taken together, these data show that Akt activation, and thus SMRT phosphorylation, decreased the sensitivity of P19 cells to retinoid-induced differentiation.

DISCUSSION

Although the RARβ2 gene is frequently down-regulated in epithelial cancers, the mechanism(s) by which this shutdown occurs remain unclear. It has been shown that a gradual down-regulation process, culminating with the recruitment of DNA methyltransferase and the establishment of a stable, repressed chromatin structure, occurs in cancer cells (10, 36). In this work, we demonstrated that hyperactivation of the PI3K/Akt signaling pathway leads to a stable recruitment of corepressor complexes to the RARβ2 promoter, causing its transcriptional down-regulation. Further support for a role of the PI3K/Akt signaling pathway in retinoid resistance comes from recently published reports. A decrease in the growth inhibitory effects of retinoids can be induced in breast cancer cell lines by overexpressing the insulin receptor substrate-1, which correlates with a hyperactivation of the PI3K/Akt pathway (37). Moreover, a subclone of the HL60 leukemia cell line with a constitutive hyperactivated PI3K/Akt pathway displayed also a retinoid-resistant phenotype (38, 39).

We demonstrated in two retinoid-responsive cell lines (P19 embryonal carcinoma and NIH3T3 fibroblasts) that histones H3 and H4 are constitutively acetylated at the RARβ2 promoter, and that this acetylation correlates with a lack of stable interaction of RARα with the corepressor SMRT. These observations are in agreement with our previously published data. In particular, we demonstrated a specific antagonist of RARα, which strengthened corepressor-RARα interaction in vitro, exhibited no effect on the acetylation
level of the RARβ2 promoter in P19 cells (26). These results strongly suggest that CoR, and more especially SMRT, does not interact or interact only very weakly with RARx in this cellular background. At this point, we are not sure whether the same phenomenon occurs for other retinoid-responsive genes. Indeed, it has been shown that the structure of the responsive elements as well as its sequence influence the ability of RXR/RAR dimers to recruit cofactors (40, 41).

Regulation of corepressor/nuclear receptor interaction by posttranslational modifications has been already reported. Phosphorylation of SMRT by the mitogen-activated protein kinase kinase 1 (MEK-1) and MEKK-1 results in a nuclear export to perinuclear and cytoplasmic compartments and, thus, to loss of repression by thyroid receptor (34). In contrast, phosphorylation of SMRT by casein kinase II increase the stability of SMRT/thyroid receptor interaction (42). We

Fig. 7. Histones H3 and H4 Acetylation, RNAP, and SMRT loading at the RARβ2 Promoter in P19 and P19 Akt-CA Subclones
A. SMRT loading at the RARβ2 promoter in P19 and Akt-CA P19 subclones. Cells were treated with 100 nM atRA for 2 h and subjected to cross-linking by 1% formaldehyde treatment. Chromatin fragments were then immunoprecipitated using antibody against SMRT and analyzed by semiquantitative PCR for the presence of the RARβ2 promoter. B. Analysis of H3 acetylation level in P19 and Akt-CA P19 subclones. Cells were subjected to cross-linking by 1% formaldehyde treatment. Chromatin fragments were then immunoprecipitated and analyzed as described in Fig. 1. Amplified DNA was quantified by densitometry, and results were expressed as the ratio Ac-H3/H3. C. Analysis of H3 and H4 acetylation levels upon retinoid treatment. P19 and Akt-CA P19 subclones were treated with 100 nM atRA for 2 h and treated with formaldehyde. CHIPs were carried out and analyzed as described in Fig. 1. D. Recruitment of RNAP in P19 and Akt-CA P19 subclones. CHIPs were carried out as described in E using an antibody against total RNAP.

Fig. 8. Impaired Retinoid Response in P19 Cells in Response to Akt Hyperactivation
Impaired neuronal differentiation in response to Akt activation. Aggregated wt P19, clones 1 and 2 were treated with the indicated concentration of atRA as described in Materials and Methods to promote neuronal differentiation. The differentiation was monitored by Western blot analysis of βIII-tubulin expression, using a polyclonal antibody. Results are expressed as arbitrary scanning units of the ratio βIII-tubulin/actin, in fold induction relative to the basal activity of wt P19, which was arbitrarily set to 1.
now show that SMRT is a direct substrate for the serine/threonine kinase Akt, and that the major Akt phosphorylation site maps to the receptor-interaction domain of the SMRT. Akt effect on RXR/RAR-SMRT association occurs most likely through a stabilization of the interaction, because SMRT is present mostly in the nucleus of P19 and HeLa cells (data not shown). In addition, inhibition of the proteasome did not modify the RXR/RAR-SMRT interaction in P19 cells (data not shown), demonstrating that SMRT phosphorylation by Akt did not increase its stability, as demonstrated for several others proteins including Mdm2 (43). It should be noted that NCoR has been reported to be phosphorylated by Akt, leading to its nuclear exclusion and loss of NCoR-mediated repression (44). However, it is unlikely that this regulation could affect RARβ2 promoter transcriptional regulation, because NCoR displays a very low affinity for RXR/RAR heterodimers as shown in Fig. 2, B and C, and as reported by others (32).

Paralleling the stabilization of RXR/RAR-SMRT interaction, overexpression of Akt-CA in P19 cells resulted in a strong decrease of the basal level of expression of the RARβ2 gene. This effect correlates with H3 and H4 deacetylation at the RARβ2 promoter. A direct relationship between histone deacetylation and transcriptional repression has been demonstrated for several promoters (45, 46). One of the suggested mechanisms is that histone deacetylation leads to a more compact chromatin structure, which prevents transcription factors access to DNA (47). In this regard, we previously demonstrated that histone acetylation at the RARβ2 promoter is necessary for RXR/RAR heterodimer binding to their responsive elements in vitro (48). In addition, we now show that histones H3 and H4 hypoacetylation, found in HeLa cells and P19-Akt CA-expressing clones, correlates with the lack of RNAP binding at the RARβ2 promoter. In addition to affecting basal transcription level, Akt-CA overexpression resulted in blunting the transcriptional response to sub saturating (<50 nM) DNA and saturation concentration (>50 nM) concentrations of atRA (Fig. 6B). Interestingly, Hazzalin and Mahadevan (49) described recently that altering histone deacetylation/acetylation dynamics at the two immediate-early genes c-fos and c-jun results in an inhibited gene transcription. As demonstrated here for the RARβ2 promoter, c-fos and c-jun promoters are constitutively trimethylated on K4 of histone H3, irrespective of their transcriptional activity. K4 methylated H3 tails are subject to a rapid turnover of acetyl group, which is required for efficient c-fos and c-jun induction. In particular, they found that blocking acetyl group turnover at these promoters, using HDAC inhibitors, results in an inhibited c-fos and c-jun expression. Interestingly, we previously demonstrated that the two HDAC inhibitors trichostatin A and sodium butyrate inhibited RARβ2 gene expression (25). It is therefore tempting to speculate that altering the dynamics of histone acetylation at the RARβ2 promoter through the recruitment of corepressor complexes also leads to a similar effect. These results further suggest that trimethylation on K4 of histone H3 is absolutely required for efficient induction of the RARβ2 gene. In this regard, we found that the RARβ2 gene expression in HeLa cells is much lower compared with wt P19 cells.

Given the importance of atRA in epithelium maintenance and differentiation, its concentration is tightly controlled. Blood concentrations of atRA ranges from 10⁻⁸ to 10⁻⁶ M and are believed to be in the nanomolar range in tissues. Thus, altering retinoid sensitivity and response of the RARβ2 promoter at these concentrations through Akt activation may be involved in the development of epithelial tumors.

The RARβ2 promoter contains a CpG island frequently methylated in cancer cells, and DNA methylation correlates with a lack of expression of the RARβ2 gene. Interestingly, Mutskov et al. (50, 51) demonstrated that maintenance of histone acetylation on a promoter is sufficient to prevent its inactivation by DNA methylation, thus emphasizing a possible role of this mechanism in counteracting RARβ2 promoter silencing. These observations further suggest that histone deacetylation at the RARβ2 promoter must occur before CpG methylation, which then acts as a secondary modification to stabilize the repressive state.

In summary, our studies provide evidence for a direct role of PI3K/Akt signaling pathway in retinoid signaling. Phosphorylation of SMRT by Akt, by regulating its ability to interact with RXR/RAR heterodimers, influences RARβ2 gene expression and subsequent biological responses to retinoids.

MATERIALS AND METHODS

Materials and Plasmids

atRA was obtained from Sigma (St. Quentin-Fallavier, France) and was reconstituted in dimethylsulfoxide. The PI3K kinase inhibitor LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzo pyran-4-one) was obtained from Cell Signaling Technology (Beverly, MA) and reconstituted in dimethylsulfoxide.

The reporter plasmids mRARβ2 promoter-luciferase/GL2, mRARβ xRARELuc, and (DR5)3-1kLuc and (Gal)3-1kLuc were described elsewhere (25, 26, 52). pS95-Gal4 SMRT was kindly provided by M. L. Privalsky (Davis, CA), VP16-NCoR and VP16-SMRT expression plasmids were kind gifts from M. Schulz (Giessen, Germany) and D. Leprince (Lille, France), respectively. pCDNA3-HA-Akt CA and pCDNA3-HA-Akt dN were kindly provided by J. R. Testa (Philadelphia, PA). SMRT mutants were constructed by site directed mutagenesis (QuikChange, Stratagene Europe, Amsterdam, The Netherlands) following the manufacturer’s protocol using VP16-SMRT or GST-SMRT (kind gift of J. D. Chen, Boston, MA) as templates. Specific oligonucleotides were used to mutate SMRT residues 933T, 1112T, 1407S, and 1434S into alanine. All constructs were verified by automated sequencing (MWG-Biotech, Roissy-CDG, France).

Cell Culture and Transfection

P19, NIH3T3, and HeLa cells were grown in DMEM Gluta max-1, high glucose (Invitrogen, Cergy-Pontoise, France)
supplemented with 10% fetal calf serum (BioWhittaker, Walkersville, MD) plus 100 U penicillin and 100 μg/ml streptomycin (Invitrogen). Transient transfection experiments were performed using the Lipofectamine 2000 reagent (Invitrogen). Luciferase assays were performed with the dual-luciferase assay system (Promega, Charbonnières, France) according to manufacturer’s guidelines. Cell viability for LY294002 experiment has been controlled using the ATP assay CellLightter-glo luminent cell viability assay (Promega) according to manufacturer’s guidelines.

To isolate stably transfected clones, P19 cells were transfected as described previously with the pcDNA3-HA-Akt CA and selected with G418 (200 μg/ml) for 10–15 d (25). Isolated clones were screened for Akt expression (see below).

Neuronal differentiation was induced as follows: P19 cells were aggregated in bacterial-grade petri dishes with the indicated concentration of aTRA after mild dissociation of adherent cells using CaCl2/MgCl2-free PBS. After 4 d, aggregates were dissociated with trypsin and replated in tissue culture dishes and allowed to differentiate for 4 more days.

RNA Preparation and Real-Time PCR

P19 cells were grown as described above and treated for the indicated time with 100 nm aTRA. Total RNA was prepared using RNAeasy Mini-kit (Qiagen, Courtaboeuf, France). Purified RNA was adjusted to 1 μg/μl, and its integrity was assessed by gel electrophoresis. Reverse transcription was performed using random hexamers as recommended by the manufacturer (Promega). cDNAs were analyzed by PCR amplification using the TaqMan PCR master mix (Applied Biosystems). The FAM/H9252/H9251 primers and probes. The 18S primers, hRAR probes were purchased from Applied Biosystems. The FAM/H9252/H9251/H9253/H9251/H9252 primer sets were optimized and selected with G418 (200 μg/ml) for 10–15 d (25). Isolated clones were screened for Akt expression (see below).

Cellular Extracts and Western Blotting

For preparation of whole P19 cell extracts, approximately 107 cells were washed twice in ice-cold PBS and scraped in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease inhibitor mixture (Sigma)]. The lysate was rotated 1 h at 4 C and centrifuged 15 min at 4 C. Preparation of nuclear and cytoplasmic extracts from P19 and HeLa cells was prepared according to Dignam et al. (53). Proteins were resolved by SDS-PAGE after boiling in SDS sample buffer and transferred onto nitrocellulose membrane (GE Healthcare, Orsay, France). Immunodetection were performed using an anti-SMRTe antiserum or anti-β-tubulin (Upstate Biotechnology) and the AP fluorescent substrate system (Promega).

Kinase Assays

Akt activities were assayed from whole-cell lysates according to the manufacturer’s protocol (Cell Signaling Technology). Briefly, cells were lysed and sonicated. Two hundred micrograms of total protein was incubated overnight at 4 C with an anti-Akt agarose-immobilized antibody. Immunoprecipitates were incubated with GST-GSK3β/β fusion protein in kinase buffer. Samples were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with an anti-phospho-GSK3β/β.

The SMRT in vitro phosphorylation assay was carried out as follows: GST-SMRT fusion proteins were expressed in Escherichia coli and immobilized on glutathione-agarose beads as described previously (48). A total of 0.5 μg GST-SMRT (wild-type or mutant) was incubated with 0.5 μg purified activated Akt or GSK3 (Upstate Biotechnology) in 50 μl kinase buffer [25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2] with 5 μCi [γ-32P]ATP during 10 min at 30 C. Kinase reactions were terminated by boiling samples in SDS sample buffer, samples were resolved on SDS-PAGE, and gel was autoradiographed.

GST-Pull-down Assays

GST-pull-down experiments were performed as described previously (54, 55).

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B.L., C.B., S.F., and P.L. have nothing to declare.

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