Characterization of the AB (AF-1) region in the muscle-specific retinoid X receptor-γ: evidence that the AF-1 region functions in a cell-specific manner

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ABSTRACT

The retinoid X receptors α-, β- and γ- (RXRs) share a highly conserved ‘C’ region or DNA binding domain (DBD). The conserved ‘DE’ region or ligand binding domain (LBD) of the RXRs is functionally complex, mediating dimerization and a ligand-dependent activation function (AF-2). The AB or N-terminal region of the RXRs is poorly conserved and encodes a ligand-independent activation function (AF-1). RXRγ mRNA is preferentially expressed in skeletal and cardiac muscle, however, cell-specific steroid receptor-mediated trans-activation is a poorly understood phenomenon. We utilized the GAL4 hybrid assay system and have demonstrated that RXRγ contains two functional domains in the AB and DE regions that activate transcription in a ligand-independent and -dependent manner respectively. The functions of the AB (AF-1) and DE (AF-2) domains were regulated by cAMP-dependent protein kinases, furthermore, the function of AF-2 in the LBD was activated by 8-Br-cAMP, independent of 9-cis-retinoic acid treatment. Deletion analysis demonstrated that the AF-1 of RXRγ is located between amino acids 1 and 103 and contained multiple motifs that were targets of cAMP-dependent protein kinases. Transfection analyses in non-muscle and myogenic cells clearly demonstrated that: (i) the AF-1 of RXRγ functions in a muscle-specific manner and is required for optimal ligand-dependent trans-activation from an RXRE; (ii) RXRγ trans-activates more efficiently in a myogenic background.

INTRODUCTION

Retinoids play an important and fundamental role in development, differentiation and homeostasis (1–3). The effects of retinoids are mediated by two subgroups of the steroid receptor superfamily of nuclear receptors that bind specific DNA sequences, termed hormone response elements (HREs), and act as ligand-inducible transcriptional regulators (4,5). The two subgroups that mediate the effects of retinoids are the retinoic acid receptors (RARα, β, γ and various isoforms) that mediate transcriptional activation in response to both all-trans-retinoic acid (RA) or 9-cis-RA and retinoid X receptors (RXRα, β and γ), that mediate transcriptional activation in response to 9-cis-RA (3,6).

The RXRs share similar structural domains with other members of the steroid receptor superfamily based on amino acid similarity (3,5). RXRs and other members of the steroid/thyroid nuclear receptor family share a highly conserved C region or DNA binding domain (DBD) and DE region or ligand binding domain (LBD) (3). The LBD of the three RXRs characterized to date is functionally complex, mediating ligand binding, dimerization and a ligand-dependent activation function (AF-2) responsible for ligand-mediated transactivation (7–11). The AB regions of the three RXRs show <40% homology (6), with RXRα and RXRγ containing an activation function (AF-1) responsible for ligand-independent transactivation (8). The functional properties of the N-terminal AB region of the steroid/thyroid receptors has developed into an area of increasing interest. Recent studies on the members of the steroid receptor gene family indicate that this region, depending on the receptor, may play an important role in DNA binding, transactivation, cell type- and/or promoter-specific regulation or interaction with the general transcription factor TFIIB (12, and references therein). In terms of the retinoid receptors transactivation and promoter-specific regulation has been shown to be mediated by the different N-terminal regions of RXRs and RARs when linked to the DBD of the estrogen receptor (8,13) or GAL4 (14).

Many of the nuclear receptors identified to date have been found to be phosphoproteins (15), including RARα, RARβ and RARγ (16–18). The protein kinase C- and cAMP-dependant protein kinase pathways have been implicated in regulation of retinoid-mediated transcription, which suggest that phosphorylation processes may be involved in regulating the function of retinoid receptors (19–21). In recent studies with RXRs and RARs it has been demonstrated that in both the presence and absence of ligand manipulation of the phosphorylation state of the cell with okadaic acid (OA), which inhibits protein phosphatases PP1 and PP2A (22), led to increases in transactivation of RXR- or RAR-responsive reporter genes in transient transfection experiments (23,24). In an effort to further investigate the role of

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phosphorylation in relation to RXRγ-mediated transcriptional activation we have studied the effects of 8-Br-cAMP [an activator of protein kinase A (PKA)] and OA on the AF-1 and AF-2 domains of RXRγ.

MATERIALS AND METHODS

Plasmids

The expression plasmids pGALO (25), pSG5 (Stratagene) and pSG5RXRγ (26) and reporter plasmids pBLCAT2 (p(CAT) (27), pG182-tkCAT (28) and G518b-CAT (29) have been described elsewhere. Generation of mRXRγ DNA fragments was performed by PCR with Pfu DNA polymerase, using the manufacturer’s buffer. All PCR products were cloned into the Smal site of pBS (Stratagene) and then isolated after EcoRI digestion. GAL-RXRγ was constructed by excising mRXRγ DNA from the pSG5-RXRγ vector, end filling with Klenow fragment and ligating into an end-filled Smal site in pGALO. GAL-RXRγAB was constructed by PCR using oligonucleotides 173, 5′-CCAGAGATCCAGGTGGAATTTAGAATGGTTTTCACAGAGAC-3′, and 225, 5′-CCAGGATTCTACAACTGGCAGCATTCTGTCAG-3′, to generate the RXRγ AB region, which was ligated into the EcoRI site of pGALO. GAL-RXRγDE was constructed by PCR using oligonucleotides 223, 5′-GCAGAATTCTACAAAGCCGGGAAGCTGTGCTA-3′, and 176, 5′-GCAGAATTCCTCGGAGTCGTGCGTTCT-3′, to generate the RXRγ DE region, which was cloned into the EcoRI site of pGALO. GAL-RXRγABDE was constructed by PCR using oligonucleotides 173 (see above) and 174, 5′-CCAGAATTCTACAAAGCCGGGAAGCTGTGCTA-3′, and 176, 5′-GCAGAATTCCTCGGAGTCGTGCGTTCT-3′, to generate the RXRγ AB and DE regions. A plasmid containing the RXRγ AB and DE regions (containing amino acids 1–138 and 205–463 of RXRγ separated by four amino acids (Glu-Glu-Phe-Thr)) in the correct orientation and reading frame was then sequenced to determine the correct orientation and reading frame. A plasmid containing the RXRγ AB and DE regions (containing amino acids 1–103 and 1–43) with 5′ reporter plasmid DNA (G518b-tkCAT, pG182-tkCAT or ptkCAT) expressing chloramphenicol acetyltransferase (CAT), mixed with the appropriate amount of expression vector (1 µg for pSG5-RXRγ, -RXRγABLBD, -RXRγAB or 3 µg for GAL-RXRγ chimera) was incubated with pUC18/carrier plasmid in each transfection experiment by the DOTAP (Boehringer Mannheim)-mediated procedure as described previously (28). The DNA/DOTAP mixture was added to the cells in 6 ml fresh medium. After a period of 24 h fresh medium with or without 0.5 mM 8-Br-cAMP, 50 nM okadaic acid and/or 9-cis-RA (10⁻⁷ M) was added to the cells. The cells were harvested for assay of CAT activity 24–72 h after the transfection period. Each transfection experiment was performed at least three times in order to overcome the variability inherent in transfections.

Mouse myogenic C2C12 cells (30,31) were grown in DMEM supplemented with 20% (v/v) FCS in 6% CO₂. Prior to and during transfection this cell line was induced to biochemically and morphologically differentiate into multinucleated myotubes by serum withdrawal in ligand-deficient medium [DMEM supplemented with 2% (v/v) charcoal-stripped FCS]. Each 60 mm dish of myogenic C2C12 cells (90–100% confluence) was transiently transfected as described above. After a period of 24 h fresh medium [DMEM supplemented with 2% (v/v) charcoal-stripped FCS] ± 9-cis-RA (10⁻⁷ M) was added to the cells. The cells were harvested for assay of CAT activity 24 h after addition of fresh medium. Each transfection experiment was performed at least three times in order to overcome the variability inherent in transfections.

CAT assays

The cells were harvested, normalized for protein concentration and CAT activity measured as previously described (32). Aliquots of the cell extracts were incubated at 37°C with 0.1–0.4 mM [¹⁴C]chloramphenicol (ICN) in the presence of 5 mM acetyl CoA and 0.25 M Tris–HCl, pH 7.8. After a 1–4 h incubation period the reaction was stopped by addition of 1 ml ethyl acetate, which was used to extract the chloramphenicol and its acetylated forms. The extracted materials were analysed on Silica gel thin layer chromatography plates as described previously (32). Quantitation of CAT assays was performed by an AMBIS β-scanner.
activated transcription from E1b TA TA box with five 17mer GAL4 binding sites linked to the CAT reporter. The system utilized CAT, containing an E1b TA TA box with five 17mer GAL4 transcription of the GAL-responsive reporter construct G5E1b-GAL4 DBD (to produce a functional regions encode modular activation domains they complement the γ ligand-independent transactivation function. GAL-RXRγABDE plasmid (which lacks the C region of RXRγ) contains the DBD), increased transcription of the G5E1b-CAT reporter which contains the LBD of RXRγ increased 9-cis-RA-mediated transcription, indicating that the AB region of RXRγ is involved in transcriptional activation we utilized the GAL4 hybrid system, whereby putative activation domains are fused to the DBD of the yeast transcription factor GAL4 (33,34). If these regions encode modular activation domains they complement the GAL4 DBD (to produce a functional trans-activator) and induce transcription of the GAL-responsive reporter construct GSE1b-CAT, containing an E1b TATA box with five 17mer GAL4 binding sites linked to the CAT reporter (29). The system utilized an SV40 promoter expression vector pGALO (25) that contains a multiple cloning site downstream of the GAL4 DBD. We fused RXRγ and various domains (e.g. AB or DE regions) of RXRγ to the GAL4 DNA binding domain and examined the ability of these chimeras to regulate expression of the GSE1b-CAT reporter in COS-1 cells. The GAL-RXRγ chimera containing the full open reading frame (ORF) of RXRγ in the presence of 9-cis-RA activated transcription ~4-fold above the control, pGALO (GAL4 DBD), and GAL-RXRγ in the absence of 9-cis-RA. This demonstrated that the GAL-RXRγ chimera conferred appropriate 9-cis-RA-dependent trans-activation via RXRγ to the GAL4 DBD (Fig. 1). The GAL-RXRγDB plasmid, which contains only the AB regions of RXRγ, with the DBD and LBD deleted, increased transcription of the reporter construct ~14-fold over the GAL DBD alone, independent of 9-cis-RA treatment (Fig. 1). This indicated that the AB region of RXRγ contained a ligand-independent transactivation function. GAL-RXRγDB, which contains the LBD of RXRγ with the AB and C regions deleted, activated transcription of the GSE1b-CAT reporter ~8-fold in a 9-cis-RA-dependent manner. The GAL-RXRγABDE plasmid (which lacks the C region of RXRγ which contains the DBD), increased transcription ~10-fold in the presence of 9-cis-RA. Deletion of only the C region of RXRγ increased 9-cis-RA-mediated activation by 2- to 4-fold in comparison with the full-length RXRγ linked to the GAL DBD (GAL-RXRγ). The lower activity of full-length RXRγ may be attributed to the presence of two DNA binding domains in the GAL-RXRγ chimeraic protein, causing possible steric hindrance in either the DNA binding or transactivation function. Possible repression of chimeric constructs containing two DBDs with the GAL system have been previously reported (14). These experiments indicate that there are two domains involved in transactivation by RXRγ, AF-1 in the AB domain, which is ligand-independent, and the ligand-dependant AF-2 in the DE domain, which activates transcription in response to the ligand 9-cis-RA.

The activity of the AF-1 and AF-2 domains of RXRγ are regulated by 8-Br-cAMP and OA

Recent reports have indicated that transcriptional activation by RXRs can be regulated by phosphorylation (23,24). We therefore investigated the affect of 8-Br-cAMP (a stimulator of cAMP-dependent protein kinases) and OA (an inhibitor of serine-threonine protein phosphatases) on the AF-1 and AF-2 functions of RXRγ. In control studies (to examine non-specific effects of 8-Br-cAMP on the GAL4 hybrid system) when COS-1 cells were transfected with the reporter plasmid GSE1b-CAT the presence of 0.5 mM 8-Br-cAMP increased CAT expression by 1.4±0.25-fold (data not shown). Furthermore, the presence of 0.5 mM 8-Br-cAMP increased CAT expression from the reporter in the presence of the GAL4 DBD (pGALO) by 2.4-fold (Fig. 2A). The ability of GAL-RXRγAB to trans-activate gene expression (in a ligand-independent manner) was increased ~4.0-fold by 8-Br-cAMP treatment (Fig. 2A). GAL-RXRγDBE increased expression of the reporter by 10-fold in the presence of 8-Br-cAMP (and more importantly, in the absence of the ligand 9-cis-RA). Activation of GAL-RXRγDBE in the presence of 8-Br-cAMP was 1.2- to 2-fold greater than that seen in GAL-RXRγDBE-transfected cells treated only with 9-cis-RA. Co-treatment of GAL-RXRγDBE with 9-cis-RA and 8-Br-cAMP resulted in a similar activation of as shown. The mean CAT activity values and standard deviations (bars) were derived from a triplicate experiment.

Figure 1. RXRγ has two regions involved in transactivation. The GAL fusion constructs containing RXRγ or various sub-domains of RXRγ (3 μg) were transfected together with the GAL reporter GSE1b-CAT (5 μg) into COS-1 cells in either the absence (−) or presence (+) of 9-cis-RA (10−7 M) (see Materials and Methods for transient transfection and CAT assay details). A schematic diagram of the GAL fusion constructs (not to scale) and a representative autoradiogram of the CAT assay of these transfections is shown. The mean CAT activity values and standard deviations (bars) were derived from a triplicate experiment.
Therefore, we cannot determine or make firm statements about the effect of OA on the reporter construct and the GAL4 DBD. Co-treatment of GAL-RXRγDE with 9-cis-RA resulted in a 4.6- and 4-fold increase of CAT activity compared with treatment with OA alone (Fig. 2 B). However, this was 2-fold less than that seen in cells treated only with 9-cis-RA and similar to the increased activity of the GAL 4 DBD after OA treatment. Co-treatment of GAL-RXRγDE with 9-cis-RA and OA resulted in a 4.5-fold greater activation of gene expression compared with treatment with either 9-cis-RA or OA. For GAL-RXRγABDE results similar to GAL-RXRγDE were observed when cells were treated with either OA and/or 9-cis-RA. Our controls demonstrated that it was difficult to interpret the specific effects of OA on RXRγ in the GAL4 hybrid system, because of the generalized effect of OA on the reporter construct and the GAL4 DBD. Therefore, we cannot determine or make firm statements about the role of serine-threonine phosphorylation in the activity of RXRγ, however, we note that OA can activate the LBD in a ligand-independent manner.

The N-terminal AF-1 of RXRγ is located between amino acids 1 and 103

We have shown that the N-terminus or AB domain of RXRγ contains a ligand-independent activation function (AF-1) located between amino acids 1 and 138 (Fig. 1). In order to further characterize the AF-1 region of RXRγ we have constructed various deletions of the AB region and fused these sub-domains to the GAL DBD (Fig. 3 A). These constructs were transfected into COS-1 cells in the absence of ligand and assayed with respect to the ability to trans-activate the reporter (Fig. 3B). A construct (GAL-RXRγ1–103) containing the first 103 amino acids of the 138 amino acid AB region of RXRγ increased activation 10.3-fold above the control, pGALO (GAL-DBD) alone, and had similar activity to the entire AB domain of RXRγ (GAL-RXRγ-decoAB). The plasmid GAL-RXRγ104–138 did not trans-activate gene expression in this assay system. This and the previous experiment demonstrate that amino acids 104–138 are not essential for activity of the AB region and do not contain an activation domain.

The constructs GAL-RXRγ1–43, GAL-RXRγ44–77 and GAL-RXRγ77–103 all activated the reporter ~2-fold, indicating that these sub-domains synergistically interacted within the context of the AB region to activate gene expression ~10-fold. The plasmid GAL-RXRγ77–138 had similar activity to GAL-RXRγ77–103, further demonstrating that amino acids 104–138 do not contain an activation domain. The constructs GAL-RXRγ44–103 and GAL-RXRγ44–138 activated gene expression 3–4-fold, indicating that the sub-domains between 44–77 and 77–103 could interact additively, but not synergistically. In summary, the data demonstrate that the AF-1 domain contains...
Figure 3. AF-1 of RXRγ is located between amino acids 1 and 103. (A) A schematic diagram of the GAL fusion constructs containing various deletions of the AB region of RXRγ are shown. The names of the various constructs represent the first and last amino acid (aa) or the beginning/end of internal deletions in the AB region of RXRγ (B) The GAL fusion constructs containing the AB region or various deletions of the AB region of RXRγ were transfected together with the GAL reporter GSE1b-CAT into COS-1 cells, as described in Figure 1, in the absence of ligand (see Materials and Methods for transient transfection and CAT assay details). A representative autoradiogram of the CAT assay of these transfections is shown. The mean CAT activity values and standard deviations (bars) were derived from a triplicate experiment.

multiple motifs in the first 103 amino acids that function synergistically to activate transcription in a ligand-independent manner.

We then examined the effect of 8-Br-cAMP and OA treatment on the various sub-domains of the AB region to identify potential regions that were targets of phosphorylation (Fig. 4). In this experiment 8-Br-cAMP and OA treatment increased the ability of the GAL DBD to trans-activate gene expression by 2- and 2.8-fold respectively. Although all the GAL-AB sub-domain chimeras were activated by OA treatment, the level of induction was <3-fold, making it difficult to make firm statements about the effects of OA on the AB region. However, the effects of 8-Br-cAMP on the AB region were quite striking in comparison. GAL-RXRγ1–103 was 5.5-fold more active in the presence of 8-Br-cAMP. The plasmid GAL-RXRγ104–138 was only 2-fold more active in the presence of 8-Br-cAMP, hence, with respect to the 2-fold effect of 8-Br-cAMP on the GAL DBD, this and the previous experiment demonstrate that amino acids 104–138 are not essential for the effect of 8-Br-cAMP on activity of the AB region.

The constructs GAL-RXRγ1–43, GAL-RXRγ44–77 and GAL-RXRγ77–103 were 5-, 3- and 5-fold respectively more active in the presence of 8-Br-cAMP. The plasmids GAL-RXRγ77–138 and GAL-RXRγ77–103 were similarly activated by 8-Br-cAMP treatment and further demonstrate that amino acids 104–138 are not involved in AB region phosphorylation. The constructs GAL-RXRγ44–103 and GAL-RXRγ44–138 were 6- and 4-fold more active in the presence of 8-Br-cAMP.

These experiments indicate that the AB regions contains multiple motifs in the first 103 amino acids that are targets of cAMP-dependent protein kinase cascades.

The AB (AF-1) region of RXRγ is required for optimal ligand-dependant transactivation in muscle cells and functions in a cell-specific manner: RXRγ trans-activates more efficiently in a myogenic background

We went on to examine the role of the AB region of RXRγ and the effect of phosphorylating agents in RXR-mediated transactivation from an optimal RXRE (G18) cloned into the heterologous herpes simplex virus thymidine kinase (tk) promoter (27) linked to the CAT gene in non-muscle and myogenic cells (pG182-tkCAT). We transfected both cell types because RXRγ is selectively expressed in skeletal and cardiac muscle. The G18 RXRE utilized was derived from a RXRγ binding site selection experiment and was the optimal sequence with respect to binding of RXRγ homodimers and RXR-dependent trans-activation in vivo by 9-cis-RA (28).

We investigated the ability of full-length RXRγ, RXRγ lacking the AB region (the RXRγΔAB construct contains amino acids 129–463) and RXRγ lacking the D/E region (the RXRγΔLBD construct contains amino acids 1–229) to transactivate an RXRE in non-muscle and muscle cells in the presence and absence of phosphorylating agents. We utilized the RXRγΔLBD construct as a control construct, since removal of the LBD of RXRs has been shown by Zhang et al. (10) to abolish receptor function (i.e. homodimerization, ligand binding and transactivation). Therefore, the use of plasmid RXRγΔLBD acts as a proper negative vector control and serves to highlight the contribution of endogenously expressed RXRs (35).

In control studies when COS-1 cells were transfected with the reporter plasmid ptkCAT relative CAT activity in the presence of 0.5 mM 8-Br-cAMP or 50 nM OA compared with untreated cells were 2.1 ± 0.2 and 2.9 ± 0.18 respectively (data not shown). COS-1 cells (Fig. 5) and C2C12 muscle cells (Fig. 6) were co-transfected with the expression vector RXRγΔLBD, RXRγΔAB or RXRγ and the reporter plasmid pG182-tkCAT. Transfection of reporter plasmid pG182-tkCAT with an expression vector containing the RXRγΔLBD construct in the presence of 9-cis-RA and/or 8-Br-cAMP or OA (in COS-1 cells) trans-activated gene expression 1.6- to 2.3-fold and negligibly in COS-1 and C2C12 cells respectively (Figs 5 and 6). These experiments in COS-1 cells and C2C12 cells verified the inability of RXR lacking the LBD to trans-activate gene expression. When cells were co-transfected with full-length RXRγ and the reporter pG182-tkCAT addition of 9-cis-RA induced a 7.2-fold increase in CAT expression in COS-1 cells (Fig. 5). After treatment with either 8-Br-cAMP or OA CAT expression was increased only 2.8-
Figure 4. Various deletions of the AB region of RXRγ are augmented by 8-Br-cAMP. The GAL fusion constructs containing the AB region or various deletions of the AB region of RXRγ were transfected together with the GAL reporter GSE1b-CAT into COS-1 cells, as described in Figure 1, in the absence (−) or presence (+) of 0.5 mM 8-Br-cAMP or 50 nM OA (see Materials and Methods for transient transfection and CAT assay details). A representative autoradiogram of the CAT assay of these transfections is shown. The mean CAT activity values and standard deviations (bars) were derived from a triplicate experiment.


d and 1.8-fold respectively. However, these increases in activation were not significant, as 8-Br-cAMP and OA stimulated CAT expression mediated by RXRγΔLBD ~2-fold (Fig. 5). Simultaneous 9-cis-RA + 8-Br-cAMP or 9-cis-RA + OA treatment stimulated CAT expression mediated by RXRγ 13.7- and 13.2-fold respectively (Fig. 5). Whether this truly reflects a synergistic activation or simply a generalized/indirect increase mediated by the non-specific effects of 8-Br-cAMP and OA on transcription in COS-1 cells is unclear. Co-transfection with RXRγΔAB and the reporter pG182-tkCAT and addition of 9-cis-RA induced a 5.9-fold increase in CAT expression. Co-treatment with 9-cis-RA and 8-Br-cAMP or OA resulted in 14.2- and 8.8-fold increases respectively (Fig. 5). These studies in COS-1 cells indicate that RXRγΔAB and RXRγ trans-activate gene expression in a similar manner. These results are in agreement with studies by Nagpal et al. (13), which showed that co-transfection of RXRγ with the AB domain removed (RXRγΔAB) did not affect activation of an RXRE (DR-1) placed upstream of the tk promoter in COS-1 cells.

Cell specificity has been found to play an important role in the activation functions (AFs) of the AB domain in the estrogen, glucocorticoid and progesterone receptors and RARs (14,36). The ability of different AFs to function has been found to: (i) vary in relation to the cell line used; (ii) depend on the spatio-temporal expression pattern of the specific receptor. This indicates that cell-specific activation mechanisms are involved in functioning of the different AFs. The RXRγ isoform is preferentially/abundantly expressed in skeletal and cardiac muscle (6,37).

Hence, we investigated whether the AF-1 and AF-2 domains of RXRγ activate/function in a cell-specific fashion. C2C12 myogenic cells were co-transfected with receptor expression vector RXRγΔLBD, RXRγΔAB or RXRγ and the reporter plasmid pG182-tkCAT in the presence and absence of 9-cis-RA (Fig. 6).

As expected, RXRγΔLBD was unable to activate pG182-tkCAT in the presence or absence of 9-cis-RA. However, full-length RXRγ produced a 19-fold induction of the pG182-tkCAT reporter in a 9-cis-RA-dependent manner (Fig. 6). This 19-fold induction of gene expression by RXRγ in muscle cells was significantly more efficient than the 7-fold induction by RXRγ in COS-1 cells (Fig. 5). In contrast to the observations in COS-1 cells, activation of the reporter by the RXRγΔAB construct after 9-cis-RA treatment was significantly less than that mediated by the native

Figure 5. The effect of 8-Br-cAMP and OA on RXR-mediated transcription. Different pSG5-RXRγ constructs, RXRγΔLBD containing amino acids 1–229 of RXRγ, RXRγΔAB containing amino acids 129–463 of RXRγ or full-length RXRγ (1 µg) were transfected into COS-1 cells together with the reporter pG182-tkCAT (5 µg) containing an optimal RXRE inserted upstream of the herpes simplex virus thymidine kinase (tk) promoter linked to the CAT gene in either the absence (−) or presence (+) of 9-cis-RA (10⁻⁷ M), 0.5 mM 8-Br-cAMP or 50 nM OA (see Materials and Methods for transient transfection and CAT assay details). Representative autoradiograms for each CAT assay of these transfections is shown. The mean CAT activity values and standard deviations (bars) were derived from a triplicate experiment.
kinase pathways are involved in retinoid-mediated transcription and reported that protein kinase C- and cAMP-dependent protein
trans-activation of the modular AF-1 and AF-2 domains of RXR chimeras to assess the ability of phosphorylation to modulate
functions in a ligand-independent manner. Although several studies have identified two separate domains involved in transactivation by
non-functional in the absence of the ligand, 9-
RXRγ demonstrated that the AB region contained multiple motifs in the first 103 amino acids that function synergistically to activate
transcription in a ligand-independent manner and are targets either directly or indirectly of cAMP-dependent protein kinase cascades. The AF-1 domain of RXRγ has been analysed using the GAL4 hybrid system in P19 embryonal carcinoma cells and has been shown to be located in the first 32 amino acids of the AB region (14), which is in contrast to our findings with AF-1 of RXRγ. This indicates that the AF-1 domains of RXRγ and RARβ may function by different mechanisms.
Transfection experiments in COS-1 cells revealed that simultaneous treatment with 9-cis-RA and OA or 8-Br-cAMP produced a synergistic activation of an optimal RXRE linked to tkCAT in an RXRγ-dependent manner. However, whether the effects of these agents were direct or indirect was masked by the effects of these phosphorylating agents on the basal reporter in the absence of functional receptor. During the course of this study two recent reports have indicated that OA is able to regulate the DNA binding activities and/or function (independent of ligand) of RXR and RAR (23,24). Differences between those studies and ours probably reflect the use of different response elements and/or cell lines, which have been demonstrated to influence receptor-mediated trans-activation.

**DISCUSSION**

We have shown in these studies utilizing the GAL4 hybrid system that RXRγ contains two transactivation functions, AF-1 and AF-2. The first of these is located in the N-terminal AB region (AF-1) and is constitutively active, independent of 9-cis-RA, while the second is located in the DE (LBD) region (AF-2) and functions in a ligand-dependent manner. These experiments are in agreement with previous studies by Nagpal et al. (8,13), which identified two separate domains involved in transactivation by RXRγ, by linkage to the estrogen receptor DBD. Interestingly, the GAL-RXRγABCDE and GAL-RXRγABDE constructs were non-functional in the absence of the ligand, 9-cis-RA. This indicates that in the presence of the ligand-dependent AF-2 domain the function of AF-1 (AB region) is repressed in COS-1 cells.

We examined the effect of 8-Br-cAMP and OA on GAL-RXRγ chimeras to assess the ability of phosphorylation to modulate trans-activation of the modular AF-1 and AF-2 domains of RXRγ in the GAL4 hybrid system. Although several studies have reported that protein kinase C- and cAMP-dependent protein kinase pathways are involved in retinoid-mediated transcription (19–21), our studies are the first to address specific activation of the AF-1 and AF-2 domains of RXRγ by phosphorylation. Our study has demonstrated that both the AF-1 and AF-2 domains of RXRγ are regulated either directly or indirectly by cAMP-dependent protein kinase cascades in a ligand-independent manner. By examination of the amino acid sequence of mRXRγ with respect to PKA consensus phosphorylation sites (RXS, RXXX or RXXS) we identified some putative PKA targets (e.g. RTLS, RVIT, RQRS, RAES and RSVS) in the AB and DE domains of the receptor, which we are evaluating by mutagenesis and transfection studies. Although OA activated the AF-1 (AB) and AF-2 (LBD) regions in the absence of ligand, the generalized effects of OA in the GAL hybrid system obscured the effects of this agent on receptor function. There are various mechanisms by which 8-Br-cAMP and OA could influence transcriptional activation mediated by the AF-1 and AF-2 domains of RXRγ. The AF-1 and AF-2 domains of RXRγ may be phosphorylated, with a consequent change in receptor conformation/activity, whereby the ability to interact with the transcriptional machinery and/or other accessory proteins is enhanced. The use of phosphorylation enhancing agents like 8-Br-cAMP and OA may also phosphorylate proteins in the transcriptional machinery (e.g. TF II A-J and/or TAFs), other accessory proteins and/or other protein kinase pathways, resulting in a change in their activities.

In our study deletion analysis of the AF-1 domain of RXRγ demonstrated that the AB region contained multiple motifs in the first 103 amino acids that function synergistically to activate transcription in a ligand-independent manner and are targets either directly or indirectly of cAMP-dependent protein kinase cascades. The AF-1 domain of RARβ has been shown to be located in the first 32 amino acids of the AB region (14), which is in contrast to our findings with AF-1 of RXRγ. This indicates that the AF-1 domains of RXRγ and RARβ may function by different mechanisms.

Transfection experiments in COS-1 cells revealed that simultaneous treatment with 9-cis-RA and OA or 8-Br-cAMP produced a synergistic activation of an optimal RXRE linked to tkCAT in an RXRγ-dependent manner. However, whether the effects of these agents were direct or indirect was masked by the effects of these phosphorylating agents on the basal reporter in the absence of functional receptor. During the course of this study two recent reports have indicated that OA is able to regulate the DNA binding activities and/or function (independent of ligand) of RXR and RAR (23,24). Differences between those studies and ours probably reflect the use of different response elements and/or cell lines, which have been demonstrated to influence receptor-mediated trans-activation.

Important observations of these studies were the demonstration that: (i) RXRγ trans-activated more efficiently in a myogenic background; (ii) the AB region of RXRγ functions in a cell-specific manner and is required for optimal ligand-dependent transactivation of an RXRE in muscle cells. Specifically, full-length RXRγ produced a 7.2- and 19-fold induction of G18 RXRE linked to tkCAT in COS-1 and myogenic C2C12 cells respectively. Furthermore, deletion of the AB region reduced trans-activation from 19- to 11-fold in myogenic cells, whereas in non-muscle cells the impact of this deletion on trans-activation was minimal (7- versus 5.9-fold). Nagpal et al. (13), using COS-1 cells and RXREs, demonstrated that deletion of the AB domain of RXRγ did not have any significant affect on the ability of the
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