The AF-1 and AF-2 Activating Domains of Retinoic Acid Receptor-α (RARα) and Their Phosphorylation Are Differentially Involved in Parietal Endodermal Differentiation of F9 Cells and Retinoid-Induced Expression of Target Genes

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Retinoic acid (RA) induces the differentiation of F9 cells cultured as monolayers into primitive endodermal-like cells, whereas a combination of RA and cAMP leads to parietal endodermal differentiation. In RA receptor α-null F9 cells (RAR$\alpha^{-/-}$ cells), RA still efficiently triggers RARγ-mediated primitive endodermal differentiation, but parietal endodermal differentiation is markedly delayed. To investigate the role of RARα1 activation functions AF-1 and AF-2 and of their phosphorylation sites during RA- and cAMP-induced parietal differentiation, cell lines reexpressing WT or mutated RARα1 were established in RAR$\alpha^{-/-}$ cells. We have found that the protein kinase A (PKA) phosphorylation site and the AF-2AD core (helix 12) of RARα1 are required for efficient parietal endodermal differentiation, whereas the AF-1 proline-directed kinase phosphorylation site is dispensible. Interestingly, deletion of the AF-1 activating domain (the A/B region), but not of the AF-2AD core, generates a dominant negative mutant that abrogates primitive endodermal differentiation when expressed in RAR$\alpha^{-/-}$ cells. We also show that the RARα AF-1 and AF-2 activation functions, but not their phosphorylation sites, are involved in the induction of RA-responsive genes in a differential promoter context-dependent manner. (Molecular Endocrinology 14:1398–1410, 2000

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INTRODUCTION

Retinoic acid (RA) is the most potent biologically active form of vitamin A. RA exhibits a wide range of activities and influences the proliferation and differentiation of a variety of cell types. In that context, F9 embryonal carcinoma (EC) cells represent a well established cell-autonomous model system for investigating retinoid signaling *in vivo*, as upon RA treatment and depending on culture conditions, they differentiate into three distinct cell types resembling primitive, parietal, and visceral endodermal extraembryonic cells (1–4). RA-induced differentiation of F9 EC cells is accompanied by an apoptotic response and a decrease in the rate of proliferation, as well as by the induction of expression of a number of genes (3, 5–7).

RA exerts its pleiotropic effects through two classes of nuclear ligand-dependent transregulators: the retinoic acid receptors (RARα, RARβ, and RARγ isotypes and their isoforms) activated by either all-*trans*-RA or its 9-*cis*-isomer and the retinoid X receptors (RXRα, RXRβ and RXRγ) activated by 9-*cis*-RA only (8–11). F9 cells express all RARs and RXRs (12–14), and two strategies have been used to investigate their roles in the response of F9 EC cells to RA treatment. Firstly, using homologous recombination, we engineered F9 cells in which either the RARα, the RARγ, or the RXRα gene, both the RARγ and RXRα genes or both the RARα and RXRα genes, are knocked out (6, 7, 15, 16). Secondly, wild-type (WT) and mutant F9 cells were treated with pan-RXR- and RAR isotype (α, β, or γ)-selective retinoids (7, 17–20). It was established that RARγ is indispensible for RA-induced differentiation of

F9 EC cells into primitive endoderm-like cells, whereas RARα is additionally required for efficient parietal endodermal differentiation that occurs in the presence of RA and cAMP (20), and for which primitive endodermal differentiation is a prerequisite. These studies also demonstrated that RAR/RXR heterodimers are the functional units transducing the retinoid signal *in vivo* (7, 19).

RARs and RXRs possess two transcriptional activation functions (AFs): AF-1, located in the N-terminal A/B region, and AF-2, associated with the ligand binding domain (LBD) (region E) and activated by agonistic ligands (8, 21–24). The integrity of a conserved amphipatic α-helix, referred to as the AF-2AD core, is required for AF-2 activity (23, 25–28, and references therein). The AF-2AD core is located in the C-terminal α helix of the LBD (helix 12) that is indispensible for the formation of the coactivator-binding surface generated during the LBD transconformation triggered by ligand binding (8, 29–31).

In addition, RARs and RXRs are phosphorylated in their AF-1 domain that contains sites for proline-directed kinases (20, 32, 33). In RARα1, the phosphorylated residue in the AF-1domain has been identified as serine 77 (32). Furthermore, RARα1 can be phosphorylated by PKA at serine 369 that is located in the LBD/AF-2 domain (34). Interestingly, RARα1 is phosphorylated at these two residues in F9 cells, as well as in transfected COS cells (20, 34). Similar phosphorylation sites are present in other RARs and have been shown to be phosphorylated in RARγ2, which is the major RARγ isoform in F9 cells (34, 35). The role of RARγ phosphorylation in retinoid-induced events has been studied in RAR$\gamma^{-/-}$ F9 cells, by establishing rescue cell lines reexpressing either RARγWT or RARγ mutated at its phosphorylation sites (20). The RARγ AF-1 domain and the proline-directed phosphorylation sites located in this domain were found to be required for rescuing the differentiation of F9 cells into primitive endoderm-like cells, whereas the PKA phosphorylation site was dispensible (20). As we had previously established that overexpression of RARα in RAR$\gamma^{-/-}$ cells could also restore their primitive and parietal endodermal differentiation (14), the role of RARα phosphorylation was similarly studied using overexpressed RARα mutants. We concluded that both the RARα AF-1 and AF-2 phosphorylation sites were not required for allowing overexpressed RARα to functionally replace RARγ for primitive endodermal differentiation, but they were apparently mandatory for parietal differentiation (20).

Thus, RARα and its phosphorylation sites could be selectively required for parietal endodermal differentiation. However, an unequivocal demonstration of this RARα-selective function requires to establish rescue cell lines reexpressing RARα (either WT or mutated) in RAR$\alpha^{-/-}$ F9 EC cells that can still differentiate into primitive endodermal cells (16) but whose parietal endodermal differentiation is greatly delayed (20), thus providing a model for directly analyzing the contribution of RARα AF-1 and AF-2 and of their phosphorylation sites in parietal endodermal differentiation. Furthermore, such lines also offer the possibility to study which RARα mutations may generate dominant negatives, preventing endogenous RARγ from mediating primitive endodermal differentiation. Rescue lines were therefore established with RARα mutated at either one of its phosphorylation sites, as well as with AF-1 or AF-2AD core deletion mutants. We demonstrate here that the PKA phosphorylation site and the AF-2AD core (helix 12 of the LBD) of RARα are required for parietal endodermal differentiation, whereas the AF-1 proline-directed kinase phosphorylation site is dispensible. None of the corresponding mutants behaved as dominant negatives, preventing primitive endodermal differentiation, whereas deletion of the AF-1 activating domain (the A/B region) generated such a dominant negative mutant. The present study also shows that RARα AF-1 and AF-2 are differentially implicated in the induction of RA-responsive genes, whereas their phosphorylation sites are not involved.

RESULTS

Generation of Stable Rescue Lines Expressing RARα1 Lacking Either AF-1 or the AF-2AD Core or Mutated at Either the AF-1 or the AF-2 Phosphorylation Sites

To investigate whether RARα AF-1 and AF-2 are involved in parietal endodermal differentiation, rescue lines stably expressing wild-type RARα (RARαWT line) and RARα lacking the A/B region (RARαΔAB line) or the AF-2AD core (RARαΔAF2 line) were derived from RAR$\alpha^{-/-}$ cells (see Fig. 1A). Similarly, to investigate the role played in this differentiation by phosphorylation of the AF-1 domain at the proline-directed kinases sites (serines 74 and 77) and of the AF-2 domain at the PKA site (serine 369), rescue lines stably expressing RARα mutated at these phosphorylation sites (RARαS74/77A and RARαS369A lines, respectively, see Fig. 1A), were also established.

Several clones were obtained for each transgene, and in each case the presence of the mutations was checked by sequencing complementary DNA (cDNA) fragments amplified by RT-PCR from total RNA of the corresponding line (data not shown). The expression level of RARαWT and of its mutant derivatives was determined after immunoprecipitation and Western blotting, and compared with the expression of endogenous RARα in WT F9 cells (Fig. 1B). RARαΔAB and RARαS369A were expressed in the selected rescue lines at levels similar to that of RARα in WT F9 cells (Fig. 1B, lanes 1, 6 and 8). The RARαWT and RARαΔAF2 rescue lines overexpressed the RARα protein relative to endogenous RARα (Fig. 1B, lanes 3 and 7). Two RARαS74/77A rescue cell lines were selected that expressed the RARα protein at a level either sim-

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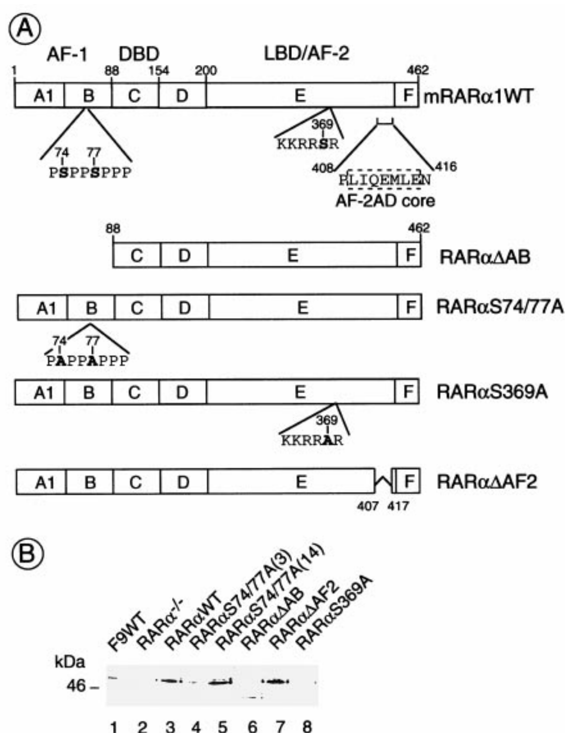

Fig. 1. Schematic Representation of the Constructs Used to Generate the Various RARα Rescue Lines in RARα$^{-/-}$ Cells

A, Mouse RARα1 with the functional domains AF-1 and AF-2, which lie in the A/B region and the E region respectively, and the DNA binding domain (DBD), are schematically represented (not to scale). The target sequences for phosphorylation by proline-directed kinases in the B domain and by protein kinase A (PKA) in the AF-2 activating domain of RARα1 are shown. The corresponding serine residues, which have been mutated to alanine (S74/77A and S369A, respectively), are indicated. The N-terminal truncated (RARαΔAB) and the AF-2AD core (amino acids 408–416) deleted (RARαΔAF2) receptors are also schematically shown. B, RARα protein in the rescue lines. Whole cell extracts were prepared from WT F9 cells, RARα$^{-/-}$ cells and each rescue line [RARαWT, RARαS74/77A (clones 3 and 14), RARαΔAB, RARαΔAF2, and RARαS369A]. RARα was immunoprecipitated with specific monoclonal antibodies [Ab9α(F)] and subsequently detected by Western blotting with a specific rabbit polyclonal antibody [RPα (F)]. The presented results correspond to a representative experiment among three.

ilar (clone 3) or higher (clone 14) than that of RARα in WT F9 cells (Fig. 1B; lanes 4 and 5).

Role of RARα AF-1 and AF-2 and of Their Phosphorylation Sites in F9 Cell Primitive and Parietal Endodermal Differentiation

When grown as monolayers in the presence of all-*trans*-RA alone, WT F9 cells differentiate at 96 h into primitive endoderm-like cells exhibiting a characteristic flat triangular morphology (4, 20). Addition of cAMP along with RA, results in the formation of parietal endoderm-like cells that have a rounded and refractile appearance (1, 20).

WT F9 cells also differentiated into primitive endoderm-like cells when treated for 96 h with a combination of a RARγ agonist (BMS961) and a pan-RXR agonist (BMS649) (36) (Fig. 2b). Parietal endodermal differentiation occurred upon subsequent addition at 48 h of the RARα agonist BMS753 (36), the pan-RXR agonist BMS649, and cAMP (Fig. 2c). No parietal differentiation occurred at 96 h (or even later at 120 h) in the absence of BMS753, confirming that RARα is required for parietal endodermal differentiation of WT F9 cells (Ref. 20 and data not shown).

As previously reported, under these conditions, primitive endodermal differentiation was not affected in RARα$^{-/-}$ cells (Fig. 2e) (16), but no parietal endodermal differentiation could be seen at 96 h (Fig. 2f) (20). In fact, parietal differentiation was markedly delayed and did not occur before 120 h (Ref. 20, and data not shown). Similar results were obtained with the RARγ agonist and cAMP, in the absence of the RARα agonist (data not shown). Thus, in RARα$^{-/-}$ cells, RARγ can substitute at least to some extent for RARα, leading to a delayed parietal endodermal differentiation.

Reexpression of RARαWT (RARαWT cell line) restored parietal endoderm differentiation at 96 h (Fig. 2i). The two RARαS74/77A lines also differentiated efficiently into parietal endoderm-like cells (Fig. 2l, and data not shown), indicating that RARα can efficiently mediate this differentiation in the absence of AF-1 phosphorylation. In contrast, the RARαS369A rescue line did not differentiate into parietal endoderm at 96 h (Fig. 2o). As in the case of the parental RARα$^{-/-}$ cell line, parietal endodermal differentiation was delayed and occurred only at 120 h (data not shown). The same observation was made with the RARαΔAF2 rescue line (Fig. 2u). Interestingly, the RARαΔAB rescue line retained a stem cell morphology in response to addition of BMS961 and BMS649. Only a few differentiated cells were observed upon subsequent addition of BMS753, BMS649, and cAMP (Fig. 2, p–r), thus suggesting that deletion of the A/B region generates a RARα dominant negative mutant that prevents endogenous RARγ from mediating primitive endodermal differentiation and subsequently suppresses parietal endodermal differentiation.

The extent of differentiation of the various rescue lines was also assessed by the expression of transcripts of two markers of primitive endodermal differentiation, collagen IV (α1), and laminin B1 (4, 37), and of one marker specific for parietal endodermal differentiation, thrombomodulin (2, 38). Semiquantitative RT-PCR analysis showed that the induction of collagen IV and laminin B1, which was not affected in RARα$^{-/-}$ cells (Fig. 3A, lanes 4–6), was similar in all rescue cell lines, except the RARαΔAB line where it was significantly reduced (Fig. 3A, lanes 16–18), in agreement with the absence of obvious morphological differentiation (see above).

No expression of thrombomodulin was seen in WT F9 cells treated with BMS961(selective for RARγ) and BMS649 (selective for all RXRs) either in the absence

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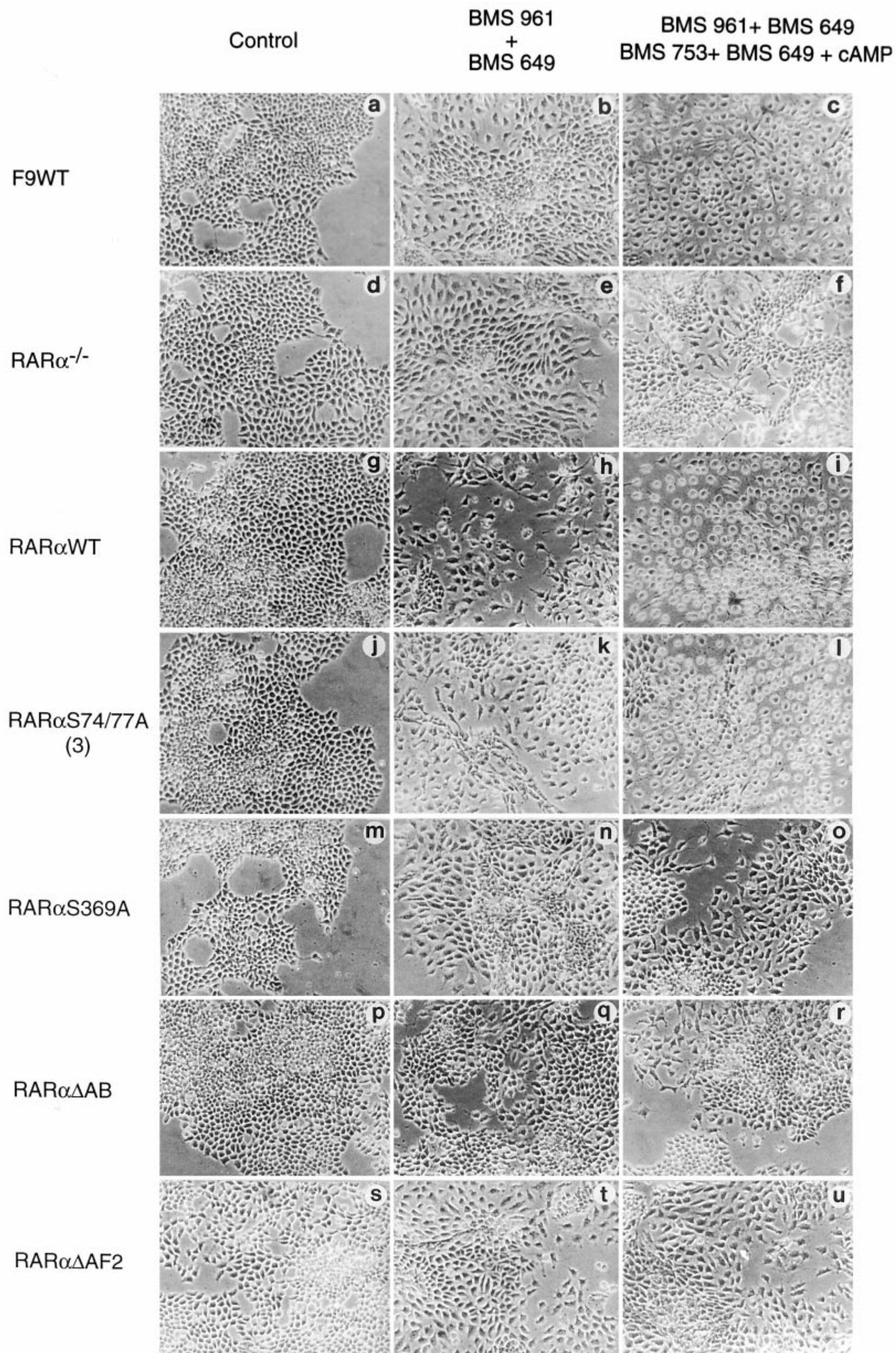

Fig. 2. The AF-2AD Core and the PKA Phosphorylation Site of RARα Are Indispensable for Rescuing Parietal Endodermal Differentiation, Whereas the Proline Kinase Site Is Not, and RAR$\alpha\Delta$AB Generates a Dominant Negative

Morphological differentiation of WT F9 cells, RAR$\alpha^{-/-}$ cells and rescue lines (as indicated) grown for 96 h in the presence of 100 nM BMS961 and 1 μM BMS649 without or with a subsequent addition at 48 h of 100 nM BMS753, 1 μM BMS649, and 250 μM cAMP as viewed under phase contrast microscopy. Control cells treated with either 0.1% ethanol (vehicle) or 250 μM cAMP remained undifferentiated.

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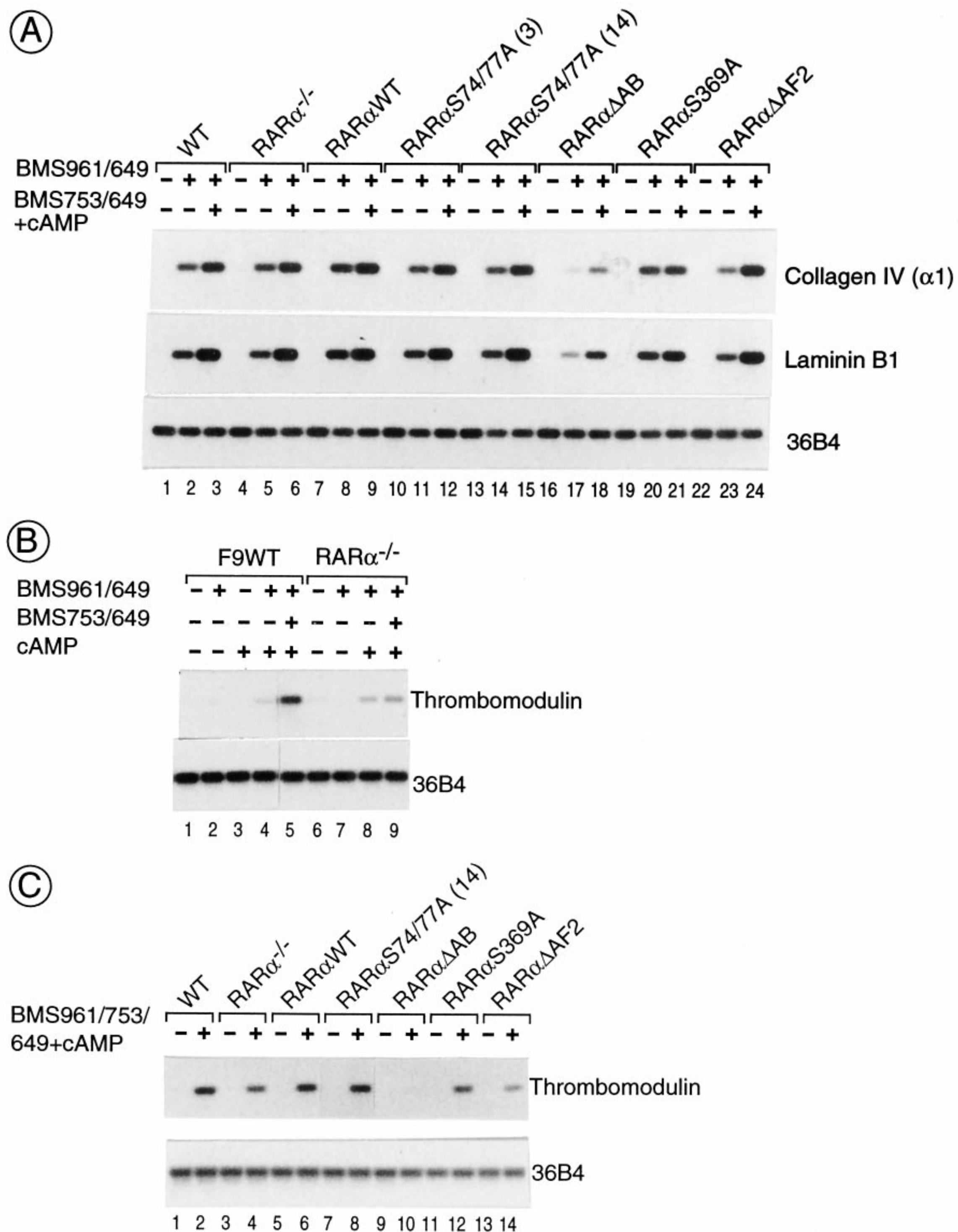

Fig. 3. Expression of the Differentiation Markers Collagen IV (α1), Laminin B1, and Thrombomodulin

A, Total RNA (2 μg) from WT cells, RAR$\alpha^{-/-}$ cells and the rescue lines treated as in Fig. 2 for 96 h was subjected to RT-PCR analysis for collagen IV (13 amplification cycles) and laminin B1 (15 amplification cycles) using transcripts of the 36B4 gene (15 amplification cycles) as an internal control to normalize the amounts of RNA. B, Total RNA (2 μg) from WT and RAR$\alpha^{-/-}$ F9 cells treated for 96 h with either vehicle (lanes 1 and 6), 250 μM cAMP (lane 3), BMS961, and BMS649 without (lanes 2 and 7) or with cAMP (lanes 4 and 8), and the BMS961/649/753 and cAMP combination (lanes 5 and 9), was subjected to RT-PCR analysis for Thrombomodulin (20 cycles) and 36B4. (C) Total RNA (2 μg) from WT, RAR$\alpha^{-/-}$ and the rescue lines treated for 96 h with vehicle or the combination of BMS961 and BMS649 and the subsequent addition at 48 h of BMS753, BMS649 and cAMP were subjected to RT-PCR for thrombomodulin and 36B4. The results presented in A, B, and C correspond to a representative experiment among three.

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or presence of cAMP (Fig. 3B, lanes 2–4), thus corroborating previous reports (2, 39). However, thrombomodulin was induced upon subsequent addition of BMS753 (selective for RARα), BMS649, and cAMP (Fig. 3B, lane 5), indicating that its expression is indeed specific for parietal endodermal differentiation. Note that there was no induction by BMS753 in the absence of the RARγ agonist (data not shown). The induction of thrombomodulin was only mildly reduced in the RARα$^{-/-}$ cell line, in agreement with the delayed morphological parietal endodermal differentiation (Fig. 3B, lanes 8 and 9 and Fig. 3C, lane 4), and also confirming that RARα can be, to some extent, functionally replaced by RARγ (20). The induction of thrombomodulin was restored in the RARαWT and RARαS74/77A lines (Fig. 3C, lanes 6 and 8). Interestingly, in the RARαS369A and RARαΔAF-2 lines, the induction of this gene was mildly reduced, as in the case of the RARα$^{-/-}$ line (Fig. 3C, lanes 12 and 14) and in agreement with the delayed parietal endodermal differentiation. In contrast, the induction of thrombomodulin was completely abrogated in the RARαΔAB rescue line (Fig. 3C, lane 10), thus corroborating the lack of differentiation.

Effect of RARα AF-1 and AF-2 Activating Domains and of Their Phosphorylation Sites on Expression of RA-Responsive Genes

Whereas knockout of the RARγ gene in F9 cells results in a marked reduction of the expression of several RA-responsive genes such as Hoxa-1, HNF3α, HNF1β, Stra6, and Stra8 (19, 20), knockout of RARα had no effect on their expression (Fig. 4, compare lanes 2 and 4; Fig. 5, compare lanes 6 and 7 and Fig. 6, lane 2). In fact, only Hoxb-1 and CRABP II gene expression was significantly reduced in RARα$^{-/-}$ cells (Fig. 4, compare lanes 2 and 4; Fig. 5, compare lanes 6 and 7 and Fig. 6, lane 2) (16, 19). The expression of these RA-responsive genes was investigated in the various rescue cell lines using semiquantitative RT-PCR (for each gene, in the linear range of the PCR amplification reaction) after treatment for 24 h with 100 nM RA or a synthetic retinoid selective for RARγ (BMS961) at 100 nM. The results that are presented in Figs. 4 and 5 are summarized in Fig. 6.

The expression of Hoxb-1 and CRABPII was restored after reexpression of RARαWT (Fig. 6, lane 3). The phosphorylation site mutants for proline-directed kinases and PKA (RARαS74/77A and RARαS369A, respectively) were as efficient as RARαWT (Fig. 6, lanes 4 and 5). RARα deleted for either the AF-2AD core or the A/B region also rescued the RA-induced expression of Hoxb-1, but not that of CRABPII (Fig. 6, lanes 6 and 7).

We previously concluded that the retinoid-induced expression of Hoxb-1 and CRABPII could be mediated by both RARα-RXR and RARγ-RXR heterodimers (19). However, the expression of both genes, in WT F9 cells, was less efficiently induced by a retinoid selective for RARγ (BMS961) than by RA (Fig. 5, compare lanes 6 and 11 and Fig. 6, compare lanes 1 and 8). Interestingly, this latter retinoid was more efficient in RARα$^{-/-}$ cells (Fig. 6, compare lanes 8 and 9), indicating that the presence of RARα was to some extent preventing RARγ to mediate the induction of Hoxb-1 (19), and CRABPII. Note the down-effect of reexpressing RARαWT (Fig. 6, lane 10), which was not observed with the two deletion mutants (Fig. 6, lanes 11 and 12).

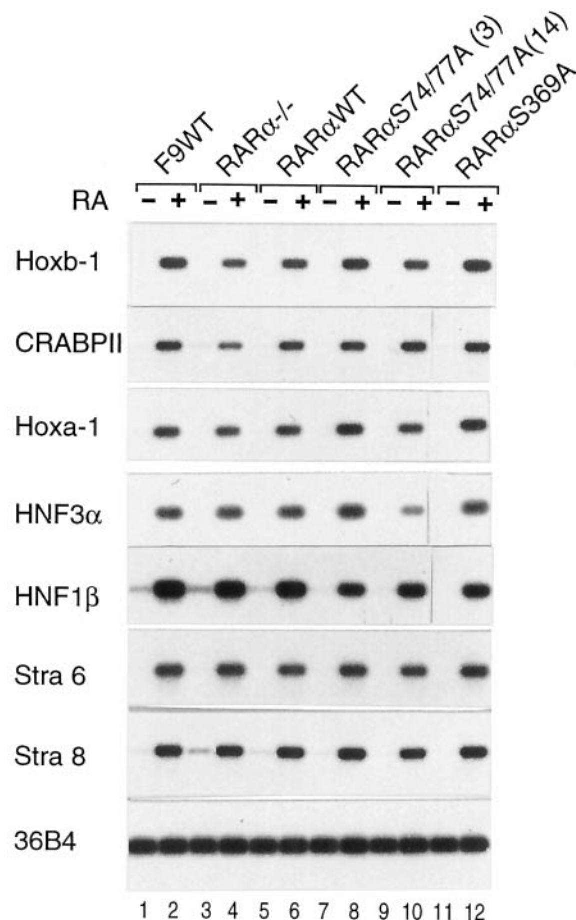

Fig. 4. The Expression or RA-Target Genes Is Not Affected in the Rescue Cell Lines Reexpressing RARα Mutated at Its Phosphorylation Sites

Total RNA was isolated from WT F9 cells, RARα$^{-/-}$ cells, and the rescue cell lines RARαWT, RARαS74/77A (clones 3 and 14) and RARαS369A with or without treatment of each cell line with 100 nM RA for 24 h as indicated. Transcripts from each gene were analyzed by semiquantitative RT-PCR, using transcripts of the 36B4 gene as an internal control to normalize the amounts of RNA. For each gene, the amount of RNA and the numbers of amplification cycles are described in *Materials and Methods*. The results correspond to a representative experiment among three.

Genes whose expression was not affected in RARα$^{-/-}$ cells, *e.g.* Hoxa-1, HNF3α, HNF1β, Stra6, and Stra8, were also analyzed in the rescue lines. Their induction was not significantly affected in RARαWT, RARαS74/77A, and RARαS369A rescue lines (Fig. 6, lanes 3–5). Note that HNF3α expression was mildly reduced in the RARαS74/77A line corresponding to

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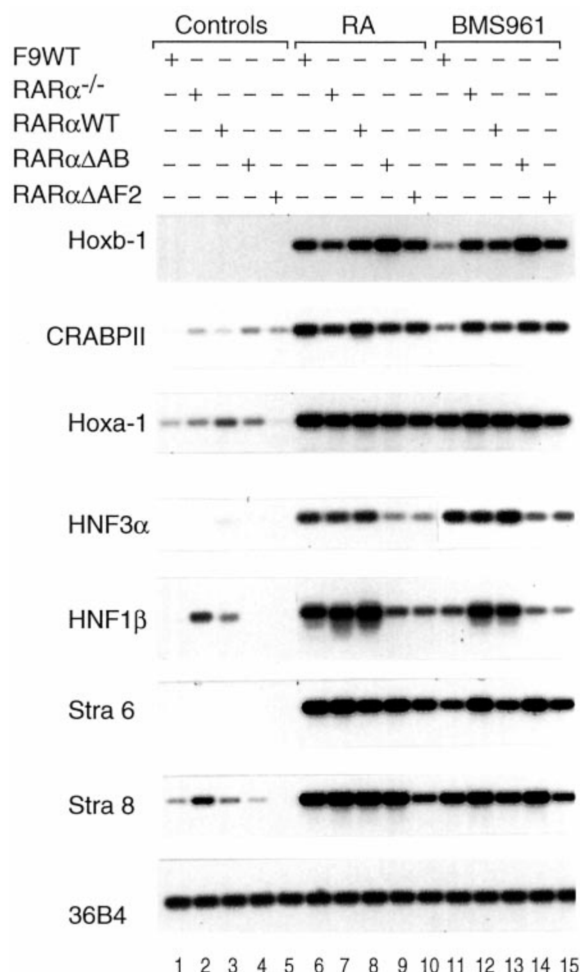

Fig. 5. Differential RA-Inducibility of RA-Target Genes in the Rescue Lines Reexpressing RARαΔAB or RARα Deleted for Its AF-2AD Core

Total RNA was isolated from WT F9 cells, RAR$\alpha^{-/-}$ cells, and the rescue cell lines RARαWT, RARαΔAB and RARαΔAF2 without (lanes 1–5) or with a 24 h treatment with 100 nM RA (lanes 6–10) or 100 nM BMS961 (lanes 11–15) as indicated. Transcripts from each gene were analyzed as in Fig. 4. The results correspond to a representative experiment among five.

clone 14 (Fig. 4, lane 10), which expressed higher RARα levels than the WT rescue line (Fig. 1B, lane 5). The RARαΔAB and RARαΔAF2 deletion mutants did not affect the RA-induced expression of Hoxa-1 but inhibited markedly those of HNF3α and HNF1β (Fig. 6, lanes 6 and 7). On the other hand, only RARαΔAF2 mildly decreased the RA-inducibility of Stra6 and Stra8 (Fig. 6, lanes 6 and 7).

The RARγ selective agonist (BMS961) was almost as efficient as RA in inducing the expression of Hoxa-1, HNF3α and Stra8 in WT and RAR$\alpha^{-/-}$ cells (Fig. 5, compare lanes 6 and 11; Fig. 6, compare lanes 1 and 8). Whereas Hoxa-1 expression was unaffected by reexpressed RARαΔAB and RARαΔAF2, that of HNF3α was reduced and that of Stra8 was reduced only by RARαΔAF2 (Fig. 6, lanes 11 and 12). Note that the basal level of expression of Stra8 was also decreased by this mutant (Fig. 5, lane 5). In contrast, the RARγ-selective retinoid was more efficient in RAR$\alpha^{-/-}$ cells than in WT F9 cells for inducing not only the expression of Stra6 (19), but also that of HNF1β (Fig. 6, lanes 8 and 9), confirming that maximal induction of some genes by RARγ may be competed for by RARα (see above). Note, in that respect, the effect of reexpressing RARαWT (Fig. 6, lane 10). Note also that in some experiments, the basal levels of HNF1β expression were mildly up-regulated in RAR$\alpha^{-/-}$ cells (Fig. 5, lane 2). RARαΔAF2 was as efficient as RARαWT in preventing maximal induction of Stra6 by RARγ but completely abrogated the activation of HNF1β by BMS961 (Fig. 6, lane 12). RARαΔAB also abrogated the activation of HNF1β but did not inhibit the activation of Stra6 by the RARγ agonist (Fig. 6, lane 11).

We also analyzed the expression of another RA-inducible gene, CYP26, whose expression has been shown to be mediated by RARγ/RXRα heterodimers (40). Accordingly, it was only slightly decreased in RAR$\alpha^{-/-}$ cells (Fig. 7, compare lanes 1–4). The induction of CYP26, which was maximal at 96 h, was not affected in the various rescue lines with the exception of the RARαΔAB line where it was markedly reduced (Fig. 7, lane 10).

DISCUSSION

The aim of the present study was to analyze the contribution of the AF-1 and AF-2 domains of RARα to F9 cell parietal endodermal differentiation and the activation of RA-responsive genes, and also to examine whether the activity of RARα could be modulated by phosphorylation of these activating domains. To that end, RARα1 mutants lacking either the AF-1 domain or the AF-2AD core (helix 12), or bearing mutations in either the AF-1 or AF-2 phosphorylation sites, were reexpressed to WT levels in RAR$\alpha^{-/-}$ cells. We demonstrate that both the AF-1 and AF-2 activating domains of RARα contribute to parietal endodermal differentiation of F9 cells and to the expression of RA-responsive genes. We also demonstrate that, whereas the phosphorylation sites located in the AF-1 activating domain are not involved in these processes, the PKA phosphorylation site located in the LBD is required for parietal endodermal differentiation. Indeed, mutation of the PKA site abrogates parietal endodermal differentiation at 96 h even in the presence of cAMP.

The RARα AF-2 Activating Domain and Its PKA Phosphorylation Site Are Required for Efficient Parietal Endodermal Differentiation

The AF-2AD core motif of RARs belongs to the C-terminal α-helix of nuclear receptor ligand binding domains (helix 12), which is indispensible for the formation of the coactivator-binding surface during the LBD transconformation triggered by ligand binding, thus generating transcriptionally active receptors (8, 31). Numerous studies

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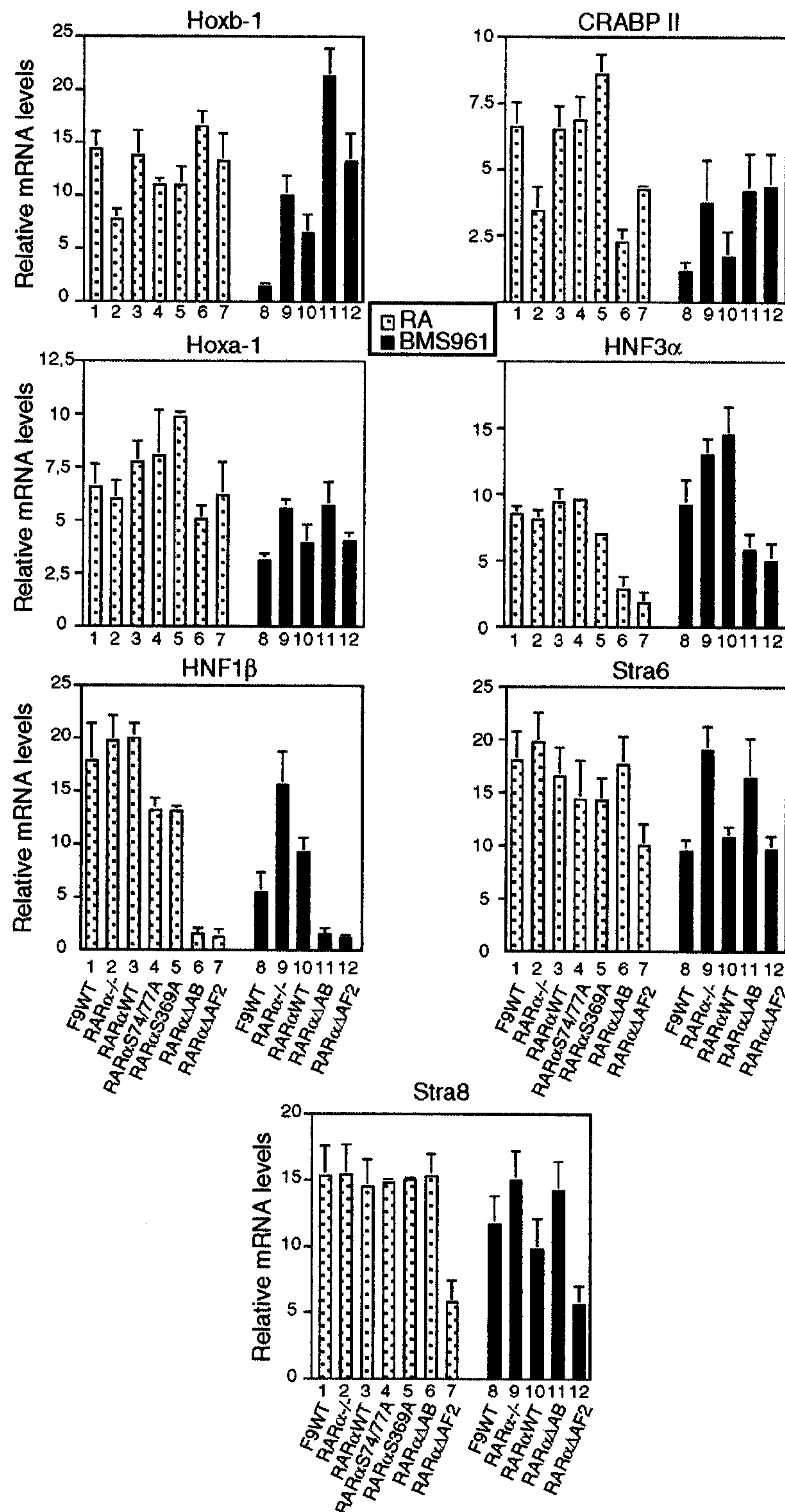

Fig. 6. Role of the AF-1 and AF-2 Activating Domains of RARα and of Their Phosphorylation in Retinoid-Induced Expression of Target Genes

The relative level of induction of RA responsive genes in WT F9 cells, RARα$^{-/-}$ cells and each of the rescue lines grown in the absence or presence of RA (100 nM, *stippled bars*) or the RARγ selective agonist (BMS961 100 nM, *solid bars*) for 24 h was estimated by semi quantitative RT-PCR as in Figs. 4 and 5, followed by quantification of the signals with a Bio-Imaging Analyzer. The values correspond to the fold-induction relative to the amount of RNA transcripts present in ethanol-treated cells, which was given an arbitrary value of 1. The values are an average ± SEM of duplicates from five independent experiments, except in the case of HNF1β where they are an average of seven experiments.

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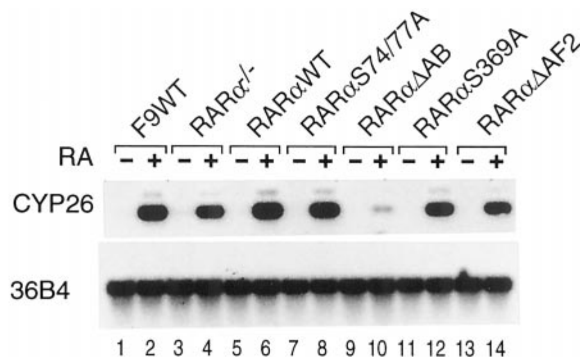

Fig. 7. The RA-Inducibility of CYP26 Is Abrogated in the RARαΔAB Rescue Cell Line

Total RNA (2 μg) from WT F9 cells, RARα$^{-/-}$ cells, and the rescue cell lines RARαWT, RARαS74/77A, RARαΔAB, RARαS369A, and RARαΔAF2 treated for 96 h with 100 nM RA was subjected to RT-PCR analysis for CYP26 (20 amplification cycles) using transcripts of the 36B4 gene as an internal control to normalize the amounts of RNA. The results correspond to a representative experiment among three.

have demonstrated that the integrity of the AF-2AD core is required for efficient ligand-dependent interactions with coactivators *in vitro* (for reviews, see Refs. 8, 41, and 42). In the present study, we demonstrate that the AF-2AD core of RARα and therefore its AF-2 activation function, is required to mediate the effects of RA and cAMP on F9 cells differentiation *in vivo*, as RARα deleted for this domain is unable to restore parietal endodermal differentiation. Note that this AF-2 activation function was also found to be required for RARγ and overexpressed RARα to restore primitive endodermal differentiation in RARγ$^{-/-}$ cells (see Table 1).

Mutation of the PKA phosphorylation site in the AF-2 domain (RARαS369A mutation) also abrogates the ability of RARα to efficiently restore parietal endodermal differentiation, demonstrating that this cAMP-dependent phosphorylation of RARα is additionally required for this differentiation process. This is in accordance with our previous report (20) showing that overexpression of RARαS369A in RARγ$^{-/-}$ cells was also unable to restore parietal endodermal differentiation (see Table 1), which suggested that this mutant exerts a dominant negative effect on endogenous RARα, preventing parietal differentiation. However, it is clear from our present results that RARαS369A has no dominant negative (dn) effect on RARγ, as the RARαS369A line normally differentiates into primitive endoderm-like cells.

Collectively these results demonstrate that the contribution of RARα to parietal endodermal differentiation of F9 cells requires the integrity of the AF-2 surface interacting with coactivators, as well as the integrity of the PKA phosphorylation site located in the AF-2 domain. The effect of this cAMP-dependent RARα LBD phosphorylation is unknown, but it may modulate the efficiency of ligand-dependent coactivator/corepressor binding to RARα, and/or its dimerization with RXR and thus its DNA binding properties to RA-response elements (34). Interestingly, PKA-mediated phosphorylation of certain coactivators may also modulate their interaction with RAR/RXR heterodimers (Refs. 41–44 and references therein).

Phosphorylation of the AF-1 Activating Domain Is Not Required for Parietal Endodermal Differentiation, but Deletion of this Domain Generates a Dominant Negative Mutant for F9 Cell Differentiation

When reexpressed in RARα$^{-/-}$ cells, RARα mutated at its proline-directed kinase phosphorylation site in the N-terminal AF-1 domain (RARαS74/77A) is as efficient as RARαWT at restoring parietal endodermal differentiation, indicating that phosphorylation of the RARα AF-1 domain is not required for this F9 cells differentiation. In contrast, we concluded in our previous study (see Table 1) that although the RARα AF-1 phosphorylation site was apparently not required to allow overexpressed RARα to restore, at the morphological level, the primitive endodermal differentiation of RARγ$^{-/-}$ cells, it was indispensible for subsequent parietal endodermal differentiation. However, the expression of several RA-responsive genes was not restored to WT levels in RARγ$^{-/-}$ cells rescued with RARαS74/77A (see Table 1). It is therefore possible that the phosphorylation of RARα AF-1 is in fact indispensible in the RARγ$^{-/-}$ rescued cells to efficiently rescue primitive endodermal differentiation at the molecular level, which is a prerequisite for subsequent parietal differentiation, whereas this phosphorylation is not required for parietal differentiation *per se*.

Expression of RARα lacking the A/B region in RARα$^{-/-}$ cells (RARαΔAB rescue line) abrogates their differentiation into primitive endoderm-like cells. This must reflect a ligand-independent (see Fig. 2) dn effect of RARαΔAB on the endogenous RARγ, which is required for primitive endodermal differentiation. This dn effect of RARαΔAB was also exerted on the expression of certain RA-responsive genes (see below and Fig. 6). Note in this respect that we have previously shown (Table 1) that RARαΔAB overexpressed in RARγ$^{-/-}$ cells could not restore RA-induced primitive endodermal differentiation and expression of most RA-induced genes, and thus could not functionally replace RARγ.

We conclude that the RARα AF-1 proline-directed phosphorylation site is dispensible for parietal endodermal differentiation of F9 cells, whereas deletion of the A/B region (see Fig. 2, RARαΔAB), but not of the AF-2AD core (RARαΔAF2), results in a dominant negative receptor that blocks the RARγ-mediated primitive endodermal differentiation.

Differential Promoter Context-Dependent Effect of the AF-1 and AF-2 Activating Domains of RARα and of Their Phosphorylation Sites on Expression of RA-Responsive Genes

Among the F9 cell RA-responsive genes that we have studied here and previously (16, 19), the expression of

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Table 1. Morphological Differentiation and Relative Expression of RA-Responsive Genes in F9 WT Cells, RARγ−/−, and RARα−/− Cells and Mutant "Rescue" Lines

F9 cell lines		RA primitive differentiation	RA+cAMP parietal differentiation	Relative expression of RA-responsive genes					
				Hoxa-1	HNF3α	HNF1β	Stra6	Hoxb-1	CRABPII
	WT	+	+	6.5 ± 1.1	8.4 ± 0.5	17.8 ± 3	17.9 ± 2.7	14.3 ± 1.6	6.6 ± 0.9
	RARγ−/−	−	−	2.6 ± 0.1	1.9 ± 0.8	0.3 ± 0.1	9.5 ± 0.4	9.9 ± 2.2	1.3 ± 0.04
	RARα−/−	+	−	5.9 ± 0.8	8.1 ± 0.7	19.6 ± 2.4	19.7 ± 2.7	7.7 ± 0.9	3.4 ± 0.9
RARγ−/− rescued by RARγ	RARγWT	+	+	10.3 ± 1.4	9.7 ± 0.5	8.1 ± 0.9	16.2 ± 3.8	8.5 ± 0.6	5.2 ± 0.7
	RARγΔAB[a]	−	−	7.2[a]	3.0[a]	1.0[a]	2.0[a]	ND	ND
	RARγS66/68A[a]	−	−	6.0[a]	6.0[a]	1.0[a]	18.0[a]	ND	ND
	RARγS360A[a]	+	+	ND	ND	ND	ND	ND	ND
	RARγΔAF2[b]	−	−	0.2 ± 0.01	0.17 ± 0.01	0.33 ± 0.01	0.5 ± 0.1	0.7 ± 0.2	1.6 ± 0.6
RARγ−/− rescued by overexpressed RARα	RARαWT	+	+	10.4 ± 0.4	8.11 ± 0.94	11.2 ± 2.9	19.4 ± 5.1	7.4 ± 0.3	8.6 ± 1.19
	RARαΔAB[a]	−	−	4.0[a]	2.0[a]	2.0[a]	4.0[a]	ND	ND
	RARαS74/77A[a]	+	−	8.0[a]	3.0[a]	8.0[a]	6.0[a]	ND	ND
	RARαS369A[a]	+	−	ND	ND	ND	ND	ND	ND
	RARαΔAF2[b]	−	−	4.25 ± 1.0	0.56 ± 0.04	0.33 ± 0.01	6.6 ± 0.3	4.45 ± 1.2	1.9 ± 0.09
RARα−/− rescued by RARα	RARαWT	+	+	7.7 ± 1.0	9.3 ± 1.0	19.9 ± 1.4	16.5 ± 2.8	13.7 ± 2.3	6.5 ± 0.9
	RARαΔAB[c]	−	−	5.1 ± 0.6	2.8 ± 0.9	1.4 ± 0.8	17.6 ± 2.7	16.5 ± 1.5	2.2 ± 0.9
	RARαS74/77A[c]	+	+	8.0 ± 2.1	9.5 ± 0.1	13.2 ± 1.0	14.3 ± 3.7	10.1 ± 0.7	6.8 ± 0.5
	RARαS369A[c]	+	−	9.8 ± 0.2	7.0 ± 0.2	13.1 ± 0.5	14.2 ± 2.1	10.1 ± 1.7	8.6 ± 0.03
	RARαΔAF2[c]	+	−	6.1 ± 1.6	1.8 ± 0.8	1.2 ± 0.7	9.9 ± 3.0	13.1 ± 2.7	4.2 ± 0.7

The extent of morphological differentiation of the WT F9 cells, RARγ−/− and RARα−/− cells and the rescue lines was estimated after 96 h of growth in the presence of RA (100 nM) or a combination of RA and cAMP as indicated. In all cases, the results are representative of three to seven independent experiments.
The relative level of induction of Hoxa-1, HNF3α, HNF1β, Stra6, Hoxb-1, and CRABPII in each cell line grown in the absence or presence of RA (100 nM) for 24 h was estimated by semiquantitative RT-PCR followed by quantification of the signals with a Bio-Imaging Analyser. The results correspond to the fold induction relative to the amount of RNA transcripts present in ethanol-treated cells which was given an arbitrary value of 1.

[a] The values are an average of at least three independent experiments which agreed within ±15% (see Ref. 20).

[b] The values are an average ± SEM of at least three experiments (see Ref. 46).

[c] The values are an average ± SEM of duplicates from three to seven independent experiments (see Figs. 4–6).

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only two of them (Hoxb-1 and CRABPII) is reduced in RAR$\alpha^{-/-}$ cells, whereas that of the others is not affected. In the present study, we have investigated the role played by RARα AF-1 and AF-2 activating domains and their phosphorylation sites in the expression of these RA-responsive genes. Our results, summarized in Fig. 6 and in Table 1, lead to the following conclusions.

Firstly, the PKA site located in the AF-2 domain and the proline-directed kinase site located in the AF-1 domain are not required for restoring the RA-induced expression of Hoxb-1 and CRABPII genes, which is decreased in RAR$\alpha^{-/-}$ cells. This is in contrast with our previous observation showing that in RAR$\gamma^{-/-}$ cells overexpressing RARα, the phosphorylation of RARα1 AF-1 (RARαS74/77A) is required for RA-induced expression of some other RA-responsive genes (see Table 1). Note in this respect that phosphorylation of RARγ AF-1 (RARγS66/68A) is also required for RA-induced expression of a subset of responsive genes (see Table 1).

Secondly, the AF-1 domain and the AF-2AD core of RARα are differentially required for RA-induced expression of target genes. Indeed, RARα deleted for either of these two domains restores the induction of Hoxb-1, but not of CRABPII. Note that a similar promoter-context dependent requirement of the AF-1 activating domains of RARα and RARγ was also reported for RA-induced expression of other responsive genes in RAR$\gamma^{-/-}$ rescue cells (see Table 1).

Thirdly, both AF-1 and AF-2 deletion mutants of RARα behave as dominant negatives in a promoter context-dependent manner, as the induction of RA-responsive genes whose expression is not altered in RAR$\alpha^{-/-}$ cells (Hoxa-1, HNF3α, HNF1β, Stra6, Stra8, and CYP26) was differentially decreased upon reexpression of RARα mutants deleted for either the A/B region or the AF-2AD core (see RAR$\alpha\Delta$AB and RAR$\alpha\Delta$AF2 in Fig. 6). Note that promoter context-dependent dn effects have also been observed with reexpressed RAR$\gamma\Delta$AF2 and overexpressed RAR$\alpha\Delta$AF2 in RAR$\gamma^{-/-}$ cells (see Table 1 and Ref. 46), in keeping with the previous suggestion that AF-2 deletion mutants may behave as dominant negatives for the transcription of RA-responsive genes (23, 45).

Fourthly, depending also on the promoter context, the presence of RARα differentially hinders the potential ability of RARγ to mediate RA-induced expression of a number of responsive genes. Indeed, in several cases, the RARγ-selective agonist BMS961 was found to be a more efficient inducing ligand in RAR$\alpha^{-/-}$ cells than in F9 WT cells (Fig. 6). These observations confirm that functional redundancies between RARs can be artefactually generated upon gene knockout (18, 20), and furthermore clearly show that the extent of these redundancies is variable, being dependent on the promoter context of the RA-responsive genes.

Conclusion

Using the cell autonomous RA-responsive F9 EC cell differentiation system, we have demonstrated in our present and previous studies (see Table 1) that the AF-1 and AF-2 activation functions of both RARα and RARγ, as well as their proline-directed kinase and PKA phosphorylated forms, play important and distinct roles in primitive and parietal endodermal differentiation and RA-induced expression of responsive genes. Moreover, our data show that RARα and RARγ AF-1 and AF-2, and their phosphorylated forms exhibit distinct functions that are differentially required, in a promoter context-dependent manner, to elicit the induction of RA-responsive genes expression. Interestingly, the dominant negative effect of some AF-1 and AF-2 deletion mutants on RARα- and RARγ-mediated induction of expression of RA-responsive genes also exhibits a promoter-context dependency. Finally, a similar promoter-context dependency is also characteristic of the artefactual functional redundancy generated between RARγ and RARα upon RARα knockout in F9 cells. All of these promoter-context dependencies certainly reflect the diversity of the responsive complexes that can be combinatorially assembled on polymorphic control regions with different receptor isoforms that are differentially phosphorylated, interact with different coactivators, and synergize with a variety of transregulators (41, 42).

MATERIALS AND METHODS

Plasmid Constructs

All constructs containing the cDNA of either mouse (m) full-length RARα1 (RARαWT), A/B region-truncated RARα (RAR$\alpha\Delta$AB), AF-2AD core-deleted RARα (RAR$\alpha\Delta$AF2), or RARα harboring mutations in phosphorylation sites were cloned into the pD402A vector (gift of D. Lohnes, Institut de Recherches Cliniques de Montréal, Montreal, Canada) which is driven by the PGK promoter (47). The expression construct pD403A containing mRARα1 cDNA cloned in pD402A, has been described (14). RAR$\alpha\Delta$AB, RARαS74/77A, and RARαS369A were isolated as *Eco*RI fragments from the corresponding pSG5 constructs (22, 32, 34) and subcloned into the same site of pD402A. RARα deleted for the AF-2AD core (amino acids 408–416) was constructed in pD402A, by replacing the WT *Sac*I/*Bam*HI fragment in pD403A by the corresponding fragment containing the deletion in the described pSG5 construct (23).

Cell Culture and Establishment of Stable Rescue Lines

F9 cells were cultured as monolayers on gelatinized surfaces as described (15). For differentiation studies, 10^5 cells were cultured in a 10-cm dish, and treated with RA (100 nM) alone or in combination with cAMP (250 μM) for 96 h, with a change of media after 48 h. Cells were also treated with a combination of a RARγ agonist (BMS961, 100 nM) and a pan-RXR agonist (BMS649, 1 μM), followed by the subsequent addition at 48 h of a RARα agonist (BMS753, 100 nM), the pan-RXR agonist and cAMP. Control cells were treated with vehicle alone (0.1% ethanol final concentration). To establish rescue lines, RAR$\alpha^{-/-}$ cells were electroporated with each of the constructs displayed in Fig. 1A along with a plasmid conferring resistance to puromycin (pD503; gift of D. Lohnes), in a ratio of 10:1. After 24–36 h, cells were selected with 0.8 μg/ml of puromycin for 10 days as described (14) and analyzed for the presence and expression of the transgene by Southern and Western blotting.

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Cell Extracts, Immunoprecipitation and Immunoblotting of RARα

Whole cell extracts were prepared (48) from rescue lines grown as monolayers in the absence of RA, and RARα was immunoprecipitated with Protein A-Sepharose cross-linked with monoclonal antibodies directed to the F region of RARα [(Ab9α(F)] (49). Proteins were resolved by SDS-PAGE (10% acrylamide), electrotransferred onto nitrocellulose filters and immunoprobed with polyclonal antibodies against the F region of RARα [RPα(F)] (49), followed by peroxidase-labeled protein A and chemiluminescence detection, according to manufacturer's protocol (Amersham Pharmacia Biotech Europe GmbH, Saclay, France).

RNA Isolation and RT-PCR

RNA was isolated using the guanidinium thiocyanate method and conditions for semiquantitative RT-PCR were as described (5). The quantity of RNA used for RT-PCR in each reaction (2 μg) was normalized with 36B4 transcripts (5, 50) which are unresponsive to retinoid treatment. The RT-PCR oligonucleotides used for Hoxa-1, HNF3α, HNF1β, Stra8, laminin B1, collagen type IV (α1), Hoxb-1, CRABPII, and 36B4 were as described (6, 20). The RT-PCR primers for thrombomodulin (51) were as follows: 5′-TGGAGCATGAGTGCTTCGC-3′ and 5′-GGTGTTGTAGGTACTAGAGA-3′. The primers for CYP26 (52) were 5′-GCTCAAGCTCTGGGACCTGT-3′ and 5′-CGATCACGAGCACGTAGCAC-3′. The amplifications were done for 13 cycles (collagen IV), 15 cycles (laminin B1, Stra8, and 36B4), 18 cycles (Hoxb-1, Hoxa-1, and HNF3α), 20 cycles (thrombomodulin, CRABPII, Stra6, and CYP26) or 24 cycles (HNF1β). Aliquots were electrophoresed on a 1.5% agarose gel and samples were transferred onto Hybond N membranes. The RT-PCR blots were probed with cognate [^{32}P]-labeled cDNA fragments or with end-labeled oligonucleotide probes for thrombomodulin and CYP26. The signals were quantified using a Bio-Imaging Analyzer (BAS 2000, Fuji Photo Film Co., Ltd., Tokyo, Japan).

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