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Review

F9 embryocarcinoma cells: a cell autonomous model to study the functional selectivity of RARs and RXRs in retinoid signaling

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Summary. Mouse F9 embryocarcinoma (EC) cells constitute a well established cell-autonomous model system for investigating retinoid signaling in vitro as, depending on culture conditions, retinoic acid (RA) can induce their differentiation into either primitive, parietal or visceral extraembryonic endoderm-like cells. These RA-induced differentiations are accompanied by decreases in proliferation rates, modifications of expression of subsets of RA-target genes, and induction of apoptosis. To elucidate the roles played by the multiple retinoid receptors (RARs and RXRs) in response to RA treatments, F9 EC cells lacking one or several RARs or RXRs were engineered through homologous recombination. Mutated RARs and/or RXRs were then reexpressed in given RAR or RXR null backgrounds. WT and mutant cells were also treated with different combinations of ligands selective for RXRs and/or for each of the three RAR isotypes. These studies lead to the conclusion that most RA-induced events (e.g. primitive and visceral differentiation, growth arrest, apoptosis and activation of expression of a number of genes) are transduced by RARγ/RXRα heterodimers, whereas some other events (e.g. parietal differentiation) are mediated by RARα/RXRα heterodimers. They also demonstrate that both AF-1 and AF-2 activation functions of RARs and RXRs, as well as their phosphorylation, are differentially required in these RA-induced events. In RARγ/RXRα heterodimers, the phosphorylation of RARγ is necessary for triggering primitive differentiation, while that of RXRα is required for growth arrest. On the other hand, phosphorylation of RARα is necessary for parietal differentiation. Thus, retinoid receptors are sophisticated signal integrators that transduce not only the effects of their cognate ligands, but also those of ligands that bind to membrane receptors.

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Introduction

Retinoids, mainly retinoic acid (RA), the biologically active metabolite of Vitamin A (retinol), play crucial roles in a wide variety of biological processes including embryonic morphogenesis and organogenesis, cell proliferation, differentiation and apoptosis, homeostasis, as well as in their disorders (e.g. malignant transformation) (Blomhoff, 1994; Gudas et al., 1994; Sporn et al., 1994; Kastner et al., 1995). These pleiotropic effects are mediated through two classes of nuclear ligand-dependent transcriptional regulators, the three Retinoic Acid Receptor isotypes (RARα, ß and γ and their isoforms) and the three Retinoid X Receptor isotypes (RXRα, ß and γ and their isoforms). In vitro studies, performed with either cell-free systems or cultured cells cotransfected with vectors overexpressing the various retinoid receptors and recombinant reporter genes indicated that RARs and RXRs bind as RAR/RXR heterodimers to response elements (REs) of RA-responsive genes (Leid et al., 1992; Glass, 1994; Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995; Chambon 1996, and references therein). They also allowed the dissection of the transcriptional role of the different retinoid receptor domains (Nagpal et al., 1992, 1993; Folkers et al., 1993; Durand et al., 1994) and of their phosphorylation (Rochette-Egly et al., 1997; Bastien et al., 2000). However, such studies are far from physiological conditions both in terms of receptor concentrations and responsive reporter genes.

In vivo genetic analyses in the mouse also supported the conclusion that RAR/RXR heterodimers are the functional units transducing the retinoid signal in vivo (Kastner et al., 1995, 1997; Mascrez et al.,1998). However, the interpretation of such studies could be equivocal at the molecular level, due to difficulties in

discriminating between cell-autonomous and non-cell-autonomous events in the intact animal, and to possible functional redundancies between receptor isotypes.

These in vitro and in vivo difficulties for investigating the functional specificity of the different retinoid receptors led us to choose the F9 murine embryonal carcinoma (EC) cell line as a cell-autonomous model system for analysing retinoid signaling, under conditions which mimick, at least to some extent, physiological processes occurring during early embryogenesis. Indeed, upon RA treatment, and depending on the culture conditions, the F9 EC cell line differentiates into cells resembling either one of the three distinct extraembryonic endoderms (primitive, parietal and visceral) (Fig. 1) (for review, see Strickland, 1981; Hogan et al., 1983; Gudas et al., 1994). Retinoid-induced differentiation of F9 cells is accompanied by a decrease in proliferation rate (anti-proliferative response) (Clifford et al., 1996, and references therein), triggering of apoptosis and RA-induced variations in expression of subsets of responsive genes (Clifford et al., 1996; Chiba et al., 1997b). Importantly, F9 EC cells express all RAR and RXR isotypes, with RARα1 and RARγ2 being the main RAR isoforms (Zelent et al., 1989; Wan et al., 1994; Taneja et al., 1995). Upon RA treatment, F9 cells also express the RARß2 isoform, due to activation of the RARß2 gene promoter which contains a RA-responsive element (de Thé et al., 1990; Hu and Gudas, 1990; Taneja et al., 1995).

Two strategies have been used to investigate the possible role played by the various RARs and RXRs in the responses of F9 EC cells to RA treatment. Firstly, the genes for either RARα, RARγ, RARß2 or RXRα were disrupted by homologous recombination (Boylan et al., 1993, 1995; Clifford et al., 1996, Faria et al. 1999). Cells lacking both RARγ and RXRα, or both RARα and RXRα, were also engineered (Chiba et al., 1997a,b). Secondly, wild type (WT) and mutant F9 cells were treated with pan-RXR- and RAR isotype (α, ß or γ) - selective retinoids (Roy et al., 1995; Taneja et al., 1996; 1997; Chiba et al., 1997a,b). Then, to investigate the possible specific role of the activation functions of RARα, RARγ and RXRα, and of their phosphorylation in the retinoid-induced events, "rescue" cell lines reexpressing WT or mutant receptors were derived from RARα, RARγ or RXRα null cells (Taneja et al., 1997; Plassat et al., 2000; Rochette-Egly et al., 2000).

This review describes how these strategies established which RAR/RXR heterodimers are involved in most RA-induced cellular events. It also illustrates

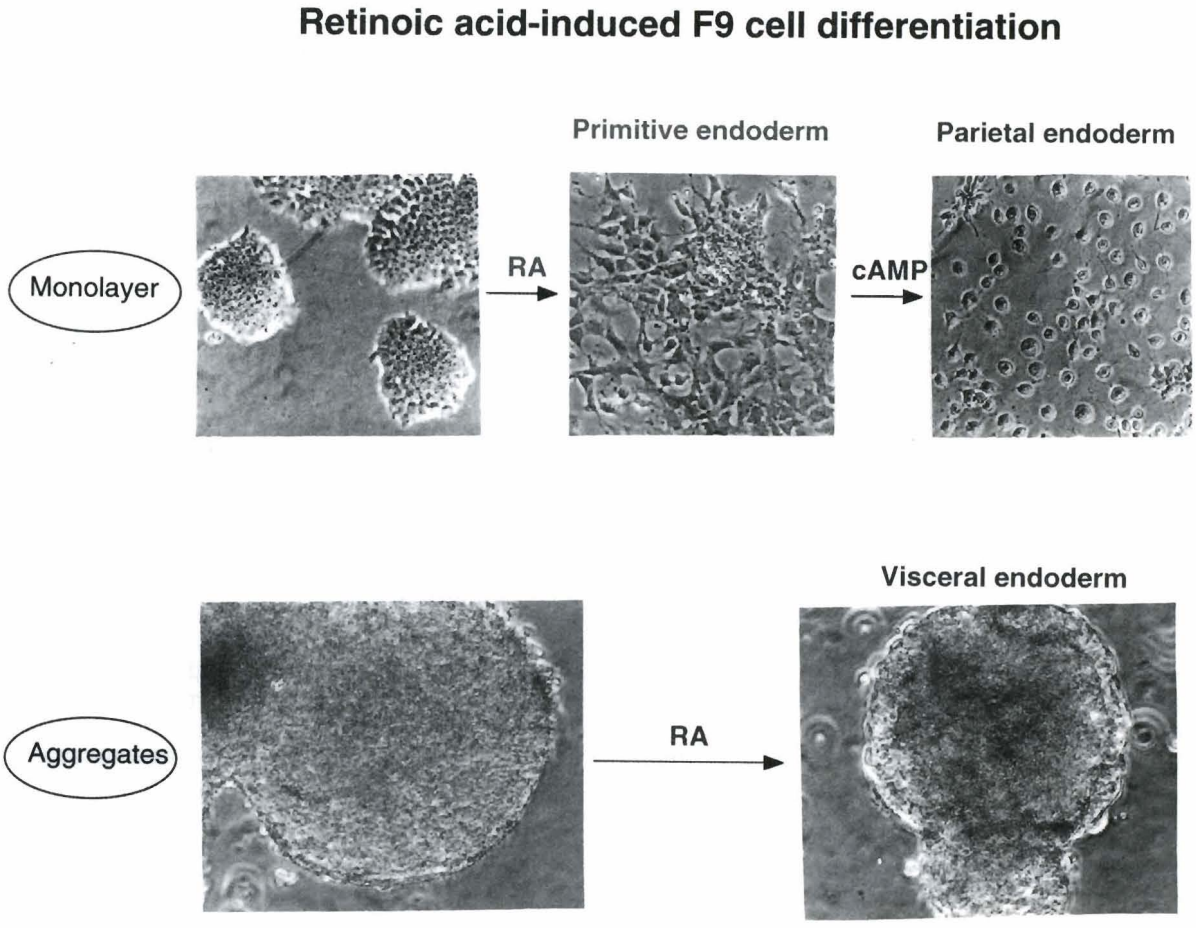

Fig. 1. Morphological differentiation of WT F9 cells into primitive endodermal-like cells when grown as monolayers in the presence of 100nM RA, into parietal endodermal-like cells when cAMP (250 μM) is added together with RA, and into visceral endodermal-like cells after a 10-day treatment of aggregates with 100nM RA.

how the "rescue" lines provided an attractive system to analyze the contribution of the different activating domains of RARs and RXRs, and of their phosphorylation in the events controlled by retinoids.

The basics of retinoid signaling

The retinoid signal is transduced by two families of nuclear receptors, the RARs and the RXRs, each consisting of three isotypes (α, ß and γ) encoded in separate genes (Leid et al., 1992; Chambon, 1996). RARs are activated by all-trans retinoic acid and its 9-cis isomer, while RXRs are only activated by 9-cis RA. For each isotype, there are at least two main isoforms which are generated by differential promoter usage and alternative splicing and differ only in their N-terminal regions (Leid et al., 1992; Mangelsdorf et al., 1995; Chambon, 1996).

RARs and RXRs are characterized by several modular domains designated A to F (see Fig. 2) (Leid et al., 1992; Chambon, 1996). The highly conserved region C corresponds to the core of the DNA-binding domain (DBD). Region E is functionally complex as it contains the ligand-binding domain (LBD), the ligand-dependent transactivation function AF-2, a dimerization domain, and also a silencing domain in the case of RARs. The activity of AF-2 is dependent on the integrity of a highly conserved amphipatic helix, the AF-2AD core that corresponds to helix 12 at the C-terminal end of the LBD (Durand et al., 1994; Mangelsdorf et al., 1995; Chambon, 1996). Ligand binding induces a major structural change in the conformation of helix 12 which creates a new surface for coactivator binding (Bourguet et al., 1995; Renaud et al., 1995; Wurtz et al., 1996; Moras and Gronemeyer, 1998; Egea et al., 2000 and references therein). The amino-terminal A/B region contains a ligand-independent transcriptional activation function AF-1 (Nagpal et al., 1992, 1993). The B region is conserved among the three RAR isotypes, but the amino-terminal A region is different for each isoform of a given RAR isotype (Leid et al., 1992). For a given RAR, the AF-1 and AF-2 activities synergize and exhibit

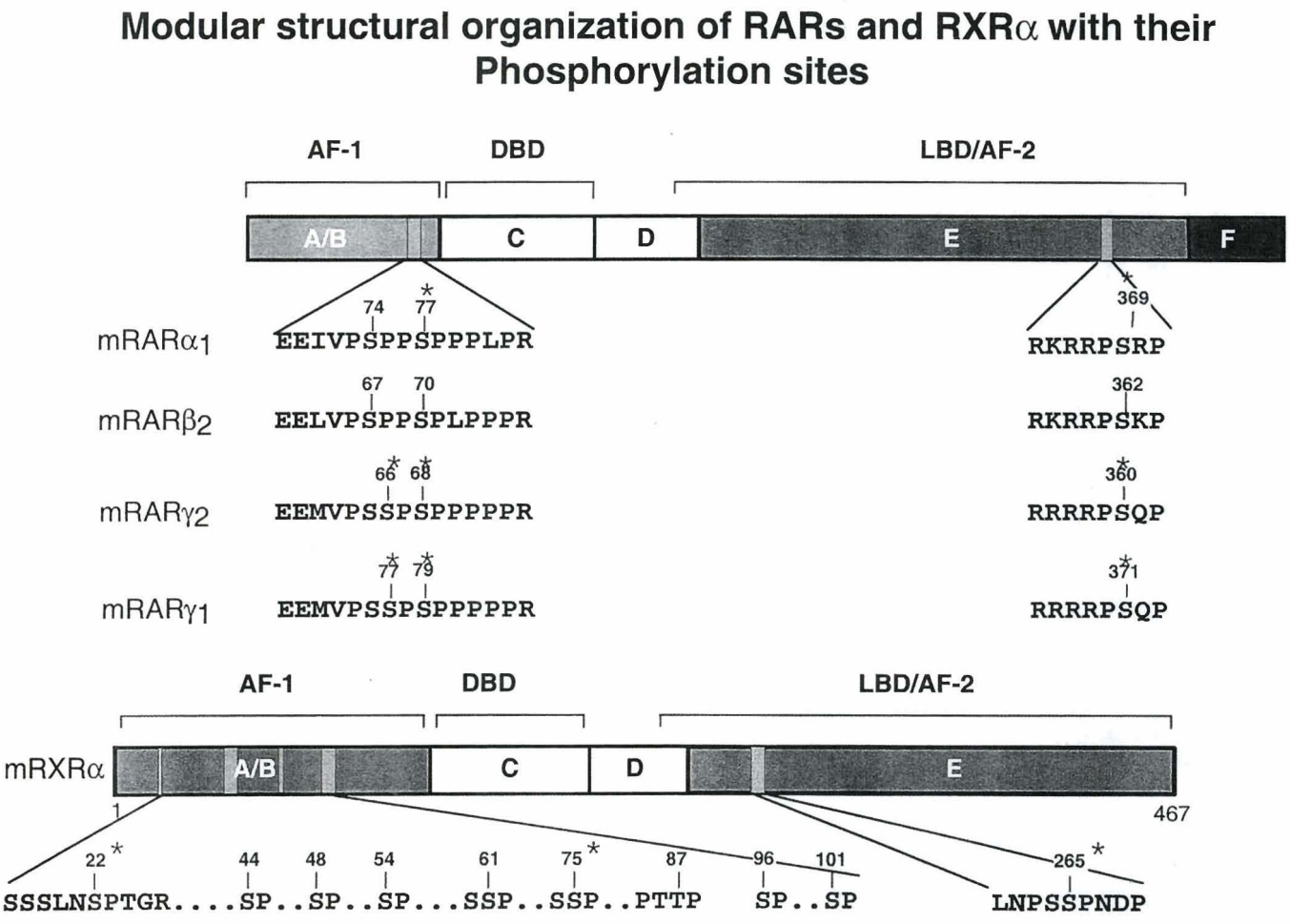

Fig. 2. Modular structure and functional organization of mouse retinoid receptors with particular emphasis on the activation functions AF-1 and AF-2. The amino acid sequence of the phosphorylation sites located in the N-terminal region of RARα, RARß, RARγ and RXRα are given and the phosphorylated serines are marked by an asterisk. The PKA sites present in the LBD are also shown.

some specificity that is dependent on both the cell type and the promoter-context of RA-responsive genes (Nagpal et al., 1992, 1993). The F region is absent in RXRs and its role in RARs, if any, is still unknown.

Like several members of the nuclear receptor superfamily, RARs and RXRs are phosphoproteins (Rochette-Egly et al., 1991, 1992; Gaub et al., 1992). The AF-1 domain contains several potential phosphorylation sites for proline-directed kinases which include the cyclin-dependent kinases (cdks) and the Mitogen-Activated Protein Kinases (MAPKs) (Morgan, 1997; Garrington and Johnson, 1999; Schaeffer and Weber, 1999). RARα and RARγ can be "constitutively" (i.e. in the absence of RA) phosphorylated in this region by the cyclin H-dependent kinase cdk7 associated to TFIIH (Rochette-Egly et al., 1997; Bastien et al., 2000), a general transcription factor also involved in DNA repair (Frit et al., 1999, and references therein). This phosphorylation which concerns serine 77 in RARα1, serines 77 and 79 in RARγ1 and serines 66 and 68 in RARγ2 (see Fig. 2), results from a retinoid-independent interaction with cdk7 and the core of TFIIH (see Fig. 3), and modulates the transcriptional properties of RARs in a promoter-context-dependent manner. RARß2 is also phosphorylated at similar residues (serines 67 and 70 in Fig. 2), but whether this receptor is phosphorylated by cdk7 remains to be determined. RARα and RARγ are also phosphorylated by Protein Kinase A at an additional residue located at the C-terminal end of region E (serine 369 in RARα1 and serine 360 in RARγ2) (Fig. 2) (Rochette-Egly et al., 1995). Finally, RXRα1 is constitutively phosphorylated in its N-terminal A region at serine 22 (Fig. 2), and can be hyperphosphorylated upon UV-activation of Stress-Activated Protein Kinases (SAPKs), namely the C-Jun N-terminal Kinases (JNK1 and JNK2) at three residues in this A/B region (S61, S75 and T87) and at one residue at the N-terminal end of the LBD (S265) (Adam-Stitah et al., 1999).

RARs and RXRs bind as RAR-RXR heterodimers to cognate DNA response elements (Fig. 4), which are composed of two directly repeated half sites [puG(G/T)TCA motif] with a spacing of 5 bp (DR5 elements), 2 bp (DR2 elements) or 1 bp (DR1 elements) (Leid et al., 1992; Mangelsdorf et al., 1995; Mangelsdorf and Evans, 1995). However, RXRs also bind to DR1 elements as homodimers, and heterodimerize not only with RARs, but also with several other nuclear receptors such as the Thyroid Hormone Receptors, the Vitamin D Receptor, the Peroxisome Proliferator Activated Receptors (PPARs), the Liver X Receptor (LXR), the Farsenoid Receptor (FXR), and a number of orphan receptors (Mangelsdorf et al., 1995; Mangelsdorf and Evans, 1995; Chambon, 1996; Giguére, 1999; Willy and Mangelsdorf, 1999). Binding of the ligand converts RAR/RXR heterodimers into strong transcriptional activators, a process that is accomplished by the release of corepressors and the subsequent association with a new set of proteins that function as transcriptional coactivators (Chambon, 1996; Moras and Gronemeyer, 1998; Glass and Rosenfeld, 2000). This results in a transcriptional competent nuclear receptor linked to the transcriptional machinery and the chromatin template (see Fig. 3). In this process, the ligand-dependent activity of RXR is subordinated to that of liganded RAR (Apfel et al., 1995; Roy et al., 1995; Botling et al., 1997; Minucci et al., 1997; Vivat et al., 1997; Willy and Mangelsdorf, 1999).

Finally, the ubiquitin-proteasome pathway appears to play a crucial role in the regulation of intracellular levels of a wide range of regulatory proteins, including nuclear receptors and other transcriptional activators, implicated in the control of key cellular functions, such as cell cycle progression, signal transduction, cell differentiation and cell death (Molinari et al., 1999; Thomas and Tyers, 2000, and references therein). As RARs and RXRs are degraded through this pathway (Zhu et al., 1999; Kopf et al., 2000), the ubiquitin-proteasome pathway might

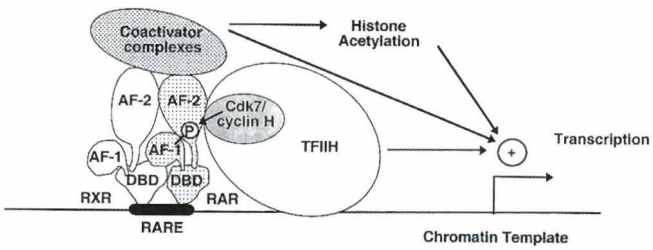

Fig. 3. Transcription by RAR/RXR heterodimers requires both RAR AF-1 phosphorylation by TFIIH, and coactivator recruitment upon ligand binding. In F9 cells, a fraction of RAR/RXR heterodimers is associated with the general transcription factor TFIIH and the cdk7 subunit of TFIIH phosphorylates the RAR AF-1 function. Upon ligand binding, RAR/RXR heterodimers bound to a RA response element (RARE) recruit coactivators (p160s, CBP/p300, etc) which allow remodelling of the chromatin template through histone acetylation.

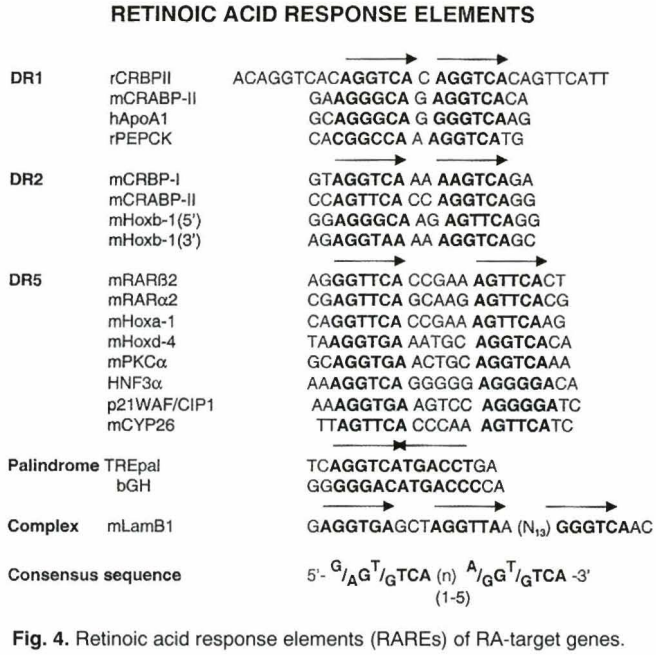

Fig. 4. Retinoic acid response elements (RAREs) of RA-target genes.

contribute to the regulation of the duration and magnitude of retinoid action

RARγ/RXRα heterodimers are functional units mediating RA-induced differentiation, growth arrest and target gene expression in F9 cells

Primitive and parietal endodermal differentiation

RA induces the differentiation of F9 cells in monolayer culture (Strickland and Mahdavi, 1978), resulting in the formation of primitive endoderm-like cells exhibiting a characteristic flat and triangular morphology (Fig. 1). Addition of cyclic AMP along with RA results in the formation of parietal endoderm-like cells (Strickland et al., 1980; Weiler-Guettler et al., 1992) which have a rounded and refractile appearance (Fig. 1).

F9 cell differentiation into primitive endodermal cells can be also induced by a RARγ-selective agonist. Moreover, a combination of the RARγ ligand present at a concentration low enough to be inactive on its own and of a pan-RXR-selective agonist, synergistically induces primitive endodermal differentiation (Roy et al., 1995; Taneja et al., 1997). However, addition of cAMP together with the RARγ-selective ligand, either alone or combined with the pan-RXR agonist, does not trigger parietal endodermal differentiation and the cells retain a primitive endoderm-like morphology (Taneja et al. 1997). The RARα-selective agonist either on its own or with cAMP, does not induce either, primitive or parietal endodermal differentiation, irrespective of the presence of the pan-RXR agonist. However, parietal endodermal differentiation occurs upon activation of both RARγ and RARα together with cAMP (Taneja et al., 1997). This synergism between the RARγ and RARα ligands was further enhanced upon concomitant activation of RXR. No or very little differentiation of F9 cells could be triggered by a RARß-selective agonist alone or associated with the pan-RXR agonist (Taneja et al., 1996). Thus, RARγ/RXRα heterodimers appear to mediate the events required for RA-induced differentiation into primitive endodermal cells, while RARα/RXRα heterodimers would play an important role in parietal endodermal differentiation in the presence of RA and cAMP. Although the activation of RARγ and RARα does not require a strict temporal order, the formation of primitive endodermal cells always precedes the appearance of parietal endoderm-like cells (Taneja et al., 1997). Thus, differentiation into primitive endoderm could be a prerequisite for parietal endodermal differentiation.

The pan-RXR ligand alone has no effect on F9 cell differentiation (Chiba et al., 1997b; Taneja et al., 1997), in agreement with the previous proposals that RXR agonists alone are unable to activate RAR/RXR heterodimers unless the RAR partner is itself liganded. Thus the RXR activity is subordinated to that of its RAR partner, as is the case in transfected cells (see above for references).

The above conclusions were definitely confirmed by knocking out the RARγ, RARα, RARß2 and RXRα genes. Knockout of either the RARγ gene (all isoforms) (Boylan et al., 1993) or the RXRα gene (Clifford et al., 1996), drastically impairs primitive and parietal differentiation. However, RARα gene knockout has milder and more restricted effects, as RARα$^{-/-}$ cells differentiate as efficiently as WT cells into primitive endoderm-like cells (Boylan et al., 1995), and are only delayed in parietal endodermal differentiation, as the corresponding morphology appeared at 120h instead of 96h (Taneja et al., 1997; Rochette-Egly et al., 2000). F9 cells lacking both RARα and RXRα still poorly differentiate into primitive and parietal endodermal cells, as do RXRα$^{-/-}$ cells. However, the double RARγ$^{-/-}$/RXRα$^{-/-}$ mutant cells are completely defective for the two types of endodermal differentiations (Chiba et al., 1997b). Surprisingly, F9 cells lacking RARß2 did not exhibit a typically differentiated morphology (Faria et al., 1999). In fact, as most of the RA-responsive genes that were studied were RA-induced in the RARß$^{-/-}$ cells following RA treatment, albeit at reduced levels, it has been suggested that RARß does not mediate the initial events in the RA-induced differentiation process, but instead would be required to achieve maximum induction (Faria et al., 1999). In other words, in F9 cells, the initial response to RA would be mediated via RARγ/RXRα heterodimers, but the subsequent large increase in RARß2 (which occurs after 16-24 hours of RA exposure) would be required for maximal morphological and molecular responses (Faria et al., 1999).

Visceral endodermal differentiation

When F9 cells are grown in suspension as aggregates (Fig. 1), low levels of RA induce a visceral endodermal phenotype in the outmost-layer of the cells resulting in an irregular surface (Strickland, 1981). Similarly to primitive endodermal differentiation, visceral endodermal differentiation also appears to be mediated by RARγ/RXRα heterodimers, as it is synergistically induced by a combination of RARγ-selective and pan-RXR-selective agonists at suboptimal concentrations (Chiba et al., 1997b). Additionally, although RXRα$^{-/-}$ cells diffferentiate efficiently (Clifford et al., 1996), RARγ$^{-/-}$ cells are delayed for visceral differentiation, and in RARγ$^{-/-}$/RXRα$^{-/-}$ cells, the surface of the aggregates remains as smooth as in untreated controls (Chiba et al., 1997b). However, both RARα$^{-/-}$ and double RARα$^{-/-}$/RXRα$^{-/-}$ mutant cell aggregates still differentiate into visceral endodermal cells (Chiba et al., 1997b). Thus, it appears that in contrast to primitive and parietal differentiation, RARγ/RXR(ß+γ) heterodimers can efficiently mediate visceral differentiation (Chiba et al., 1997b).

Growth arrest

Retinoid-induced differentiation of F9 cells is also

accompanied by a decrease in the proliferation rate (antiproliferative response), with a diminished rate of DNA synthesis and an increase in the fraction of cells in the G1 phase of the cell cycle (Clifford et al., 1996, and references therein). The functional units mediating the antiproliferative action of RA are also RARγ/RXRα heterodimers as only a RARγ-selective agonist combined to a pan-RXR-selective one was able to inhibit proliferation (Chiba et al., 1997b). Moreover, a substantial reduced antiproliferative response to RA was observed in RXRα$^{-/-}$ cells (Clifford et al., 1996) and RARγ$^{-/-}$/RXRα$^{-/-}$ cells were completely defective for growth arrest (Chiba et al., 1997b). Surprisingly, RARß2$^{-/-}$ cells did not efficiently arrest growth in response to RA (Faria et al., 1999). In fact, in this cell line, the reduced antiproliferative response has not been attributed to the absence of RARß2, but to lower levels of RXRα than in WT F9 cells (Faria et al., 1999), resulting in lower amounts of the active RARγ/RXRα heterodimer.

Activation of RA target genes

In F9 cells, a number of RA-responsive genes, such as Hoxa-1, HNF3α, HNF1ß, Stra4 and Stra6 which contain RAREs in their promoter, are controlled by RARγ/RXRα heterodimers (Taneja et al., 1996; Chiba et al., 1997a; Plassat et al., 2000; Rochette-Egly et al., 2000). In RARγ/RXRα pairs, both partners cooperate to activate transcription of these RA target genes, since a combination of the pan-RXR-selective ligand and of the RARγ-selective one at concentration low enough to be inactive on its own, synergistically induced their expression in a dose-dependent manner (Taneja et al., 1996; Chiba et al., 1997a). In contrast, RARα- and RARß-selective agonists have little or no effect when combined with the pan-RXR agonist. Moreover, the induction of these genes, which is reduced in RXRα$^{-/-}$ and RARγ$^{-/-}$ cells (but not in RARα$^{-/-}$ cells) (Clifford et al., 1996; Chiba et al., 1997a; Taneja et al., 1997), is abrogated in double RARγ$^{-/-}$/RXRα$^{-/-}$ mutant cells and not significantly further affected in RARα$^{-/-}$/RXRα$^{-/-}$ cells (Chiba et al., 1997a).

It must be noted that the activation of some other RA-target genes, although controlled by RARγ/RXRα pairs, is more complex. As an example, Hoxb-1 is activated by both RARγ/RXRα and RARα/RXRα heterodimers, as its expression is only slightly affected in RARγ$^{-/-}$ and RARα$^{-/-}$ cells (Plassat et al., 2000; Rochette-Egly et al., 2000), and is nearly abolished in cells doubly mutated for RXRα and either RARα or RARγ (Chiba et al., 1997a). In contrast, the induction of Stra8 is preferentially mediated by RARγ/RXR (ß+γ) pairs, since it is reduced in RARγ$^{-/-}$ cells, but not in RXRα$^{-/-}$ cells and is not abrogated in the double RARγ$^{-/-}$/RXRα$^{-/-}$ mutant cells (Chiba et al., 1997a). Moreover, both the RARγ- and RARα-selective retinoids, have very little effect on the expression of Hoxb-1 (Plassat et al., 2000; Rochette-Egly et al., 2000), but their combination induces Hoxb-1 expression as efficiently as does RA (Chiba et al., 1997a). Finally, the activation of RARß2 has been shown to be mediated by multiple RAR (α, ß, γ)/RXR (α, ß, γ) pairs. In this respect, although preferentially induced by a combination of RARγ- and pan-RXR-selective agonists (Taneja et al., 1996), RARß2 activation is not affected in either RARγ$^{-/-}$ or RARα$^{-/-}$ cells. In fact, it is only slightly decreased in RXRα$^{-/-}$ and RARα$^{-/-}$/RXRα$^{-/-}$ cells, and further reduced (but not abrogated) in RARγ$^{-/-}$/RXRα$^{-/-}$ cells (Chiba et al., 1997a).

All these data support the conclusion that in F9 cells, RAR/RXR heterodimers are the functional units transducing the retinoid signal, and indicate that distinct RAR/RXR isotype combinations are preferentially involved in the various RA-induced cellular events. Interestingly, in F9 cells, RARγ/RXRα heterodimers are also targets for the ubiquitin proteasome pathway, since both RARγ and RXRα (and not RARα) are ubiquitinylated and degraded following a treatment with RA (Kopf et al., 2000), or a combination of RARγ- and pan-RXR-selective agonists at suboptimal concentrations (Kopf E., Chambon P. and Rochette-Egly C., unpublished observations). Since RARγ/RXRα heterodimers are the main RA transducers in F9 cells, playing a key role in the induction of most endogenous RA-responsive genes and in the initiation of the differentiation processes, it has been proposed that the ubiquitin-proteasome pathway provides a mechanism for negative feedback regulation of RA action (Kopf et al., 2000).

Redundancies between RARs and RXRs can be artefactually generated by gene knock-outs in F9 cells

According to the studies summarized above, RARγ/RXRα heterodimers are involved in most RA-induced events in F9 cells. Moreover, a number of these events, e.g. primitive endodermal differentiation, appear to be mediated only by this pair, as RARγ and/or RXRα gene knockouts are not compensated by possible functional redundancies among RARs/RXRs heterodimers. However, in other cases, gene knockouts appear to generate artefactual conditions unmasking potential functional redundancies which are not present in WT conditions. In other words, in these cases, suppression or mutation of one RAR or RXR can be functionally compensated by other RARs or RXRs.

For example, in RARγ$^{-/-}$ cells, the RARß/pan-RXR and RARα/pan-RXR-selective agonist combinations, which have little or no effect on WT F9 cells, trigger visceral differentiation and inhibit proliferation of RARγ$^{-/-}$ cells (Chiba et al., 1997b). Thus, RARα and/or RARß can replace RARγ in these RA-induced events. Moreover, the induction of certain RA-target genes such as Hoxa-1, Hoxb-1 and RARß2 by the RARα-selective agonist is much stronger in RARγ$^{-/-}$ cells, indicating that to some extent, the presence of RARγ prevents RARα

from mediating these inductions (Taneja et al., 1996; Plassat et al., 2000).

RARα knockout also generates artefactual conditions, as RARγ could substitute for RARα in RARα$^{-/-}$ F9 cells. RARγ activation with a selective agonist, can indeed bring about a delayed parietal differentiation of RARα$^{-/-}$ cells, whereas it does not result in any parietal differentiation in WT F9 cells (Taneja et al., 1997). In addition, in the absence of RARα, the RARγ-selective ligand is more efficient for inducing growth arrest (Chiba et al., 1997b) or some RA-target genes, such as Hoxa-1, Hoxb-1, Stra6 and RARß2 (Taneja et al., 1996; Chiba et al., 1997a; Rochette-Egly et al., 2000).

Functional redundancies occur not only among RARs, but also among RXRs, as the pan-RXR-selective agonist in combination with the RARγ one can trigger efficiently visceral differentiation of RXRα$^{-/-}$ and RARα$^{-/-}$/RXRα$^{-/-}$ cells (Chiba et al., 1997b). This combination also synergistically induces the expression of the RARß2 gene in RARα$^{-/-}$/RXRα$^{-/-}$ cells (Chiba et al., 1997a). Reciprocally, in agreement with the inducibility of the RARß2 gene by multiple RAR/RXR pairs, the pan-RXR/RARα agonist combination was efficient in RARγ$^{-/-}$/RXRα$^{-/-}$ cells (Chiba et al., 1997a). Thus, in certain RA-induced cellular events, the other RXR isotypes (RXRß and RXRγ) can substitute for RXRα in cells lacking RXRα. How the presence of a RXR/RAR heterodimer prevents other potentially functionally redundant heterodimers from transducing the RA signal is unknown, but it could be related to their differential affinities for the RAREs of the target genes.

Surprisingly, these studies also revealed that the proliferation of mutant cells lacking both RARγ and RXRα was increased by RARα and RARß agonists combined with the pan-RXR agonist (Chiba et al., 1997b). Thus, either RARα/RXR (ß+γ) or RARß/RXR (ß+γ) heterodimers can mediate a proliferative effect of RA in the absence of RARγ and RXRα. Note that induction of proliferation of certain cell types by retinoids has been reported (Koshimizu et al., 1995).

In conclusion, in F9 cells, gene knock-out can generate artefactual conditions in which ablation of a given RAR or RXR can be functionally compensated by the other RARs or RXRs. Interestingly, in some events such as cell proliferation, the activation of the other RAR/RXR heterodimers can induce opposite effects.

Both AF-1 and AF-2 Activation Functions of RARγ, RARα and RXRα are necessary for the response of F9 cells to RA

To dissect the functional roles of the ligand-independent activation functions AF-1 and AF-2 in RA-induced cellular events, "rescue" lines stably expressing RARγ, RARα or RXRα lacking these AFs were established, starting from a null background (Fig. 5). As the various responses of F9 cells to RA could also be restored in RARγ$^{-/-}$ cells by overexpressing RARα (Taneja et al., 1995), rescue lines overexpressing WT or mutant RARα were also established.

This strategy allowed us to demonstrate that the AF-1 activating domain of RARs is required to transduce the RA signal (Taneja et al., 1997; Rochette-Egly et al., 2000) (see Table 1). Indeed, in RARγ$^{-/-}$ cells, reexpression of RARγ lacking the AF-1-containing N-terminal region (RARγΔAF-1) does not restore primitive endodermal differentiation and the subsequent parietal differentiation. The same results were obtained with overexpressed RARαΔAF-1. Similarly, RARαΔAF-1 was unable to restore parietal differentiation in RARα$^{-/-}$ cells. In fact, this deletion mutant behaved as a dominant negative, as it abrogated the primitive differentiation of RARα$^{-/-}$ cells (Rochette-Egly et al., 2000).

To determine whether the same phenomenom occurs

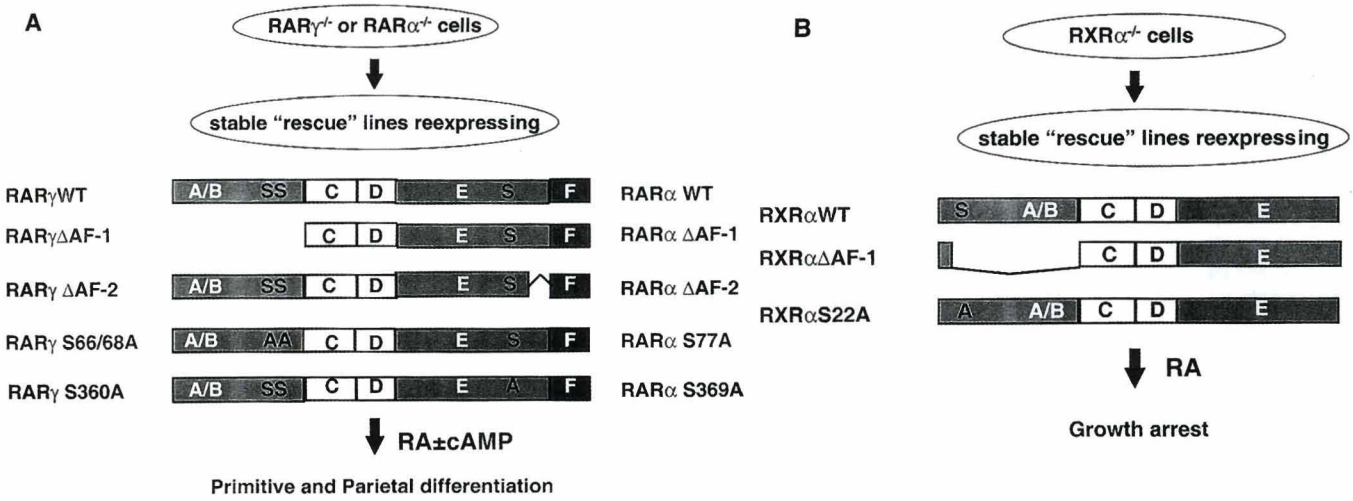

Fig. 5. Schematic representation of the mouse RAR and RXR mutants used to generate the various rescue lines. **A.** RARα and RARγ rescue lines. **B.** RXRα rescue lines.

for visceral differentiation, RARγ$^{-/-}$/RXRα$^{-/-}$ cells were rescued with either RARγWT or RARγΔAF-1 (Kopf E., Chambon P. and Rochette-Egly C., unpublished results). As expected, reexpression of RARγWT restored full visceral differentiation. However, cells reexpressing RARγΔAF-1 did not show any visceral differentiation at 4 days. In fact, morphological differentiation appeared at 10 days, indicating that this mutant brings about a delayed morphological differentiation. Altogether, these results indicate that the AF-1 domain is required for RARs to trigger F9 cell differentiation (Table 1).

Interestingly, and in contrast to that was observed with RARs, RXRαΔAF-1 rescued the differentiation of RXRα$^{-/-}$ cells as efficiently as RXRαWT (Clifford J., Chiba H., Metzger D. and Chambon P., unpublished results). However, the AF-1 activating domain of RXRα was found to be required for the antiproliferative effect of RA (Table 1) (Bastien J., Chambon P. and Rochette-Egly C., manuscript in preparation). Whether the AF-1 domain of RARγ also plays a role in this RA-induced cellular event has not been investigated as yet.

Efficient expression of most RA-inducible genes also requires the AF-1 domain. For example, in RARγ$^{-/-}$ cells, either RARγΔAF-1 or overexpressed RARαΔAF-1 are unable to restore the activation of genes such as HNF3α, HNF1ß and Stra6 (Taneja et al., 1997 and Table 2). However, it must be noted that for other RA-target genes, the contribution of the AF-1 domain depends on the receptor isotype and on the promoter context. As an example, the induction of Hoxa-1 is unaffected by the deletion of the RARγ AF-1-activating domain, whereas it is dependent on that of overexpressed RARα (Taneja et al., 1997). Interestingly, in RARα$^{-/-}$ cells, where the activation of most genes is not altered, the same RARα deletion mutant behaves as a dominant negative with a promoter-context dependency (Rochette-Egly et al., 2000 and Table 2). Whether the AF-1 activation domain of RXRα is important for the activation of RA-target genes remains to be determined.

The same strategy demonstrated that the AF-2 activating function of RARα, RARγ and RXRα is also indispensible for the RA-induced cellular events (Table 1). In this respect, neither RARγ nor overexpressed RARα deleted for their AF-2AD core (Helix 12) could restore primitive and subsequent parietal differentiation of RARγ$^{-/-}$ cells (Plassat et al., 2000). Similarly, RARα deleted for this domain was unable to restore parietal endodermal differentiation in RARα$^{-/-}$ cells (Rochette-Egly et al., 2000). These results are in agreement with the lack of differentiation of the RAC65 P19 EC cells which express a truncated form of RARα lacking the AF-2AD core (Pratt et al., 1990; Kruyt et al., 1992; Costa and Mc Burney, 1996). Finally, RXRα deleted for its AF-2AD core was also unable to restore the differentiation of RXRα$^{-/-}$ cells at limiting ligand concentrations (Clifford J., Chiba H., Metzger D. and Chambon P., unpublished results). As yet, the role of the AF-2AD core of the two partners in RARγ/RXRα heterodimers has not been studied for the antiproliferative action of RA. However, from the present data, it can be predicted that this motif contributes to the growth arrest induced by RA. Finally, the AF-2 of RARγ and RARα is required for the efficient induction of all the tested genes, since RARγΔAF-2 and overexpressed RARαΔAF-2 are inefficient in restoring their inducibility in RARγ$^{-/-}$ cells (Plassat et al., 2000; Rochette-Egly et al., 2000 and Table 2). This is in accordance with previous reports

Table 1. Involvement of the various RARs and RXRs in the transduction of the retinoid signal in F9 cells and role of their activation functions and phosphorylation sites

RETINOID-INDUCED	HETERODIME INVOLVED	ACTIVATION FUNCTIONS AND PHOSPHORYLATION SITES INVOLVED	
		RARs	RXRs
Primitive Endodermal differentiation	RARγ/RXRα (in all instances) (a,b,c)	- RARγ AF-1 and AF-2 (a, e) - RARγ phosphorylation sites in AF-1(S66 and S68) (a)	- RXRα AF-2 (f) - No role for RXRα AF-1 and phosphorylation sites (f)
Parietal endodermal differentiation	RARα/RXRα (a) - RARγ/RXRα in the absence of RARα (a) - Primitive differentiation is a prerequisite (a)	- RARα AF-1 and AF-2 (d) - RARα PKA site (in the AF-2 LBD) (d)	- RXRαAF-2 (f) - No role for RXRα AF-1 and phosphorylation site (f)
Visceral endodermal differentiation	RARγ/RXR(α, ß, γ) (b) - RAR(α,ß)/RXRα in the absence of RARγ (b) - RARγ/RXR(ß,γ) in the absence of RXRα (b)	- RARγ AF-1 *and* AF-2 (f) - RARγ AF-1 and AF-2 (f) - RARγ phosphorylation sites in AF-1 (S66 and S68) (f)	ND
Growth arrest	RARγ/RXRα (b) RAR(α,ß)/RXRα in the absence of RARγ (b)	ND	- RXRα AF-1 (f) - RXRα phosphorylation in AF-1 (S22) (f)

(a):Taneja et al., 1997; (b): Chiba et al., 1997b; (c): Clifford et al., 1996; (d): Rochette-Egly et al., 2000; (e): Plassat et al., 2000; (f): Unpublished results.

showing that the AF-2 AD core motif is an essential element of the ligand-inducible activation function AF-2 (Chambon, 1996) and cooperates with the AF-1 activating domain (Nagpal et al., 1992, 1993).

The study of the RARαΔAF-2 and RARγΔAF-2 rescue lines also confirmed the previous in vitro observations which suggested that RARs deleted for their AF-2AD core behave as dominant negatives (Damm et al., 1993; Durand et al., 1994; Schulman et al., 1996). Indeed, in the RARγ$^{-/-}$ background, RARγΔAF-2 and to a lesser extent RARαΔAF-2 inhibited the residual activation of certain genes by RARα (Plassat et al., 2000). This was particularly clear in the case of Hoxb-1 (Table 2) and RARß2 whose induction can be mediated by either RARγ/RXR or RARα/RXR heterodimers and is not affected in RARγ$^{-/-}$ cells. Similar conclusions came from results with the same RARα mutants reexpressed in RARα$^{-/-}$ cells where the induction of most RA-responsive genes is not altered. Indeed, in these cells, reexpression of RARαΔAF-2 decreased the RA-induced expression of a subset of responsive genes (Rochette-Egly et al., 2000 and Table 2).

In conclusion, this "rescue" strategy allowed us to demonstrate that RARs need the integrity of both their AF-1 and AF-2 domains to efficiently transduce the retinoid signal.

Role of RARα, RARγ and RXRα phosphorylation.

RARα and RARγ are "constitutively" phosphorylated in a ligand-independent manner in their N-terminal AF-1 domain by cdk7 within TFIIH (see Fig. 2). Since RARα1 and RARγ2 are phosphorylated in F9 cells, at one (S77) and two (S66 and S68) serine residues respectively (Rochette-Egly et al., 1997; Bastien et al., 2000), the "rescue" strategy was also used to study the role of these phosphorylations in RA-induced cellular events. Stable rescue lines reexpressing RARα and RARγ mutated at these phosphorylation sites (RARαS77A and RARγS66A/S68A cell lines) were established (Taneja et al., 1997; Rochette-Egly et al., 2000) (Fig. 5A).

Phosphorylation of serines 66 and 68 is mandatory for RARγ to restore, in RARγ$^{-/-}$ cells, primitive endodermal differentiation and the subsequent parietal differentiation (Taneja et al., 1997). In contrast, for RARα, phosphorylation of serine 77 is not required to efficiently restore the parietal endodermal differentiation of RARα$^{-/-}$ cells (Rochette-Egly et al., 2000). Thus, phosphorylation of the AF-1 activation domain of RARγ is required for primitive endodermal differentiation (Table 1), while phosphorylation of the same residues in RARα does not appear to play a role in parietal differentiation. However, in RARγ$^{-/-}$ cells, phosphorylation of overexpressed RARα was found to be mandatory for parietal endodermal differentiation, while it was not required to restore primitive endodermal differentiation at the morphological level (Taneja et al., 1997). Interestingly, several RA-responsive genes are not restored to WT levels in RARγ$^{-/-}$ cells rescued with RARαS77A (see below). Thus, RARα phosphorylation

Table 2. Relative expression of RA-responsive genes in RARγ$^{-/-}$ and RARα$^{-/-}$ cells and mutant rescue lines.

F9 cell lines	Hoxa-1	HNF3α	HNF1ß	Stra6	Hoxb-1
WT	6.5	8.4	17.8	17.9	11.3
RARγ$^{-/-}$	2.6	1.9	0.3	9.5	9.9
RARα$^{-/-}$	5.9	8.1	19.6	19.7	7.7
RARγ$^{-/-}$ rescued by RARγ (a, b)					
RARγ-WT	10.3	9.7	8.1	16.2	8.5
RARγ-ΔAF-1	7.2	3.0	1.0	2.0	ND
RARγ-S66/68A	6.0	6.0	1.0	18.0	ND
RARγ-S360A	ND	ND	ND	ND	ND
RARγ-ΔAF-2	0.2	0.17	0.33	0.5	0.7
RARγ$^{-/-}$ rescued by overexpressed RARα (a,b)					
RARαWT	10.4	8.11	11.2	19.4	7.4
RARαΔAF-1	4.0	2.0	2.0	4.0	ND
RARαS77A	8.0	3.0	8.0	6.0	ND
RARαS369A	ND	ND	ND	ND	ND
RARαΔAF-2	4.25	0.56	0.33	6.6	4.45
RARα$^{-/-}$ rescued by RARα (c)					
RARαWT	7.7	9.3	19.9	16.5	13.7
RARαΔAF-1	5.1	2.8	1.4	17.6	16.5
RARαS77A	8.0	9.5	13.2	14.3	10.1
RARαS369A	9.8	7.0	13.1	14.2	10.1
RARαΔAF-2	6.1	1.8	1.2	9.9	13.1

The relative level of induction of Hoxa-1, HNF3α, HNF1ß, Stra6 and Hoxb-1 in each cell line grown in the presence of RA (100nM) for 24 hours 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. The values are an average of at least three experiments which agreed with ±15%. (a): Taneja et al., 1997; (b): Plassat et al., 2000; (c): Rochette-Egly et al., 2000.

could be indispensible to efficiently rescue primitive endodermal differentiation, which is a prerequisite for subsequent parietal differentiation, whereas this phosphorylation would not be required for parietal differentiation per se.

RARγ is also unable to efficiently trigger visceral differentiation, unless it is phosphorylated in the A/B region (Table 1). Indeed, RARγ$^{-/-}$/RXRα$^{-/-}$ cells reexpressing RARγS66A/S68A are delayed in visceral differentiation, as morphological differentiation appears at 10 days instead of 4 days (Kopf E. and Rochette-Egly C., unpublished results).

RXRα is also "constitutively" phosphorylated, in a ligand-independent manner, in the AF-1-activating domain at serine 22 (Fig. 2). To determine the role of this phosphorylation, stable rescue lines reexpressing RXRα mutated at this phosphorylation site (RXRαS22A line) (Fig. 5B) were established in a RXRα$^{-/-}$ background. This cell line differentiates efficiently indicating that RXRα can mediate differentiation in the absence of serine 22 phosphorylation (Table 1). However, this phosphorylation site is required for the antiproliferative response to RA (Bastien J., Chambon P. and Rochette-Egly C., manuscript in preparation). Whether RARγ phosphorylation in its AF-1 domain also plays a role in the antiproliferative effect of RA and synergizes with RXRα phosphorylation to mediate the antiproliferative action of RA remains to be determined.

Previous studies performed with cells cotransfected with reporter genes and plasmids expressing the retinoid receptors, either WT or mutated at their phosphorylation sites (RARαS77A and RARγS66/68A), suggested that phosphorylation of these residues by cdk7 is important for the transactivation properties of RARs, but with a promoter-context dependency(Rochette-Egly et al., 1997; Bastien et al., 2000). Thus, it was important to determine whether similar results could be obtained with the various RA-target genes identified in F9 cells. By testing the inducibility of the RA-target genes in the RARγ$^{-/-}$ rescue lines reexpressing RARγS66/68A or overexpressing RARαS77A, the requirement for these phosphorylation sites was found to be promoter-dependent, and also receptor isotype-dependent (Taneja et al., 1997). As an example, phosphorylation of RARγ is required for the efficient induction of HNF1ß and to a lesser extent of HNF3α, but not for the other genes tested, while phosphorylation of overexpressed RARα is necessary for the induction of HNF3α and Stra6 (Table 2). Thus, it would be interesting to determine whether phosphorylation of RXRα at serine 22 in the A/B region is important for the activation of certain RA target genes and/or cooperates with that of RARs.

Finally, it has been demonstrated that the degradation of RARγ by the ubiquitin-proteasome pathway requires phosphorylation of the receptor (Kopf et al., 2000). However, since cell lines reexpressing RARγS66A/S68A do not differentiate upon RA treatment, it cannot be concluded whether the defect in the degradation of this mutant receptor reflects the lack of phosphorylation or is a consequence of the absence of differentiation.

RARγ and RARα are also phosphorylated by PKA at a conserved residue located at the C-terminal end of the LBD (S360 for RARγ2 and S369 for RARα1). Phosphorylation of the PKA site of RARγ was found to be dispensible for both primitive and parietal differentiation. However, phosphorylation of this site in RARα is mandatory to rescue parietal differentiation either in RARα$^{-/-}$ cells, or when overexpressed in RARγ$^{-/-}$ cells (Taneja et al., 1997). Thus, it was concluded that phosphorylation of the PKA site of RARα is required for parietal differentiation (Table 1). However, none of the RA-responsive genes tested so far were found to be regulated by phosphorylation of RARs at their PKA site (Rochette-Egly et al., 2000). Studies are in progress in order to identify genes whose expression is sensitive to such phosphorylations.

Conclusions and perspectives

F9 cells represent an interesting autonomous cell system to study the molecular mechanisms underlying the complex biological events induced by retinoids, since they respond to retinoic acid by a morphological differentiation, a decrease in the proliferation rate and the activation of subsets of RA-target genes. Moreover, as F9 cells also respond to cyclic AMP, they constitute an excellent system to study crosstalk between the retinoid and other signaling pathways, including the PKA pathway.

To elucidate the molecular mechanisms of retinoid signaling in this mammalian cell-autonomous system,

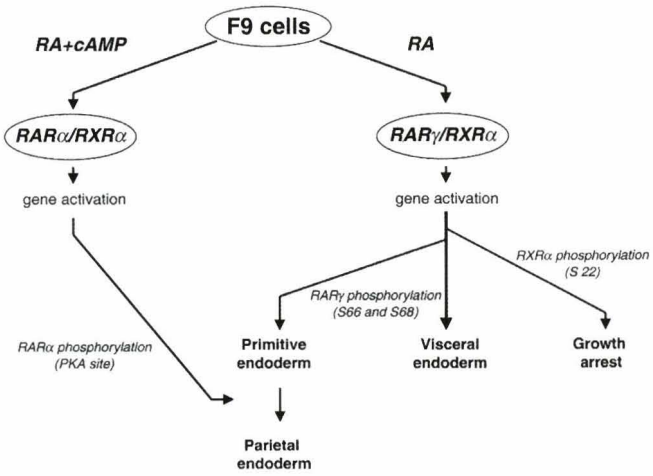

Fig. 6. In RA-treated F9 cells, the activation of most RA-target genes, the induction of primitive and visceral differentiation and the growth arrest involve RARγ/RXRα heterodimers. In these heterodimers, the phosphorylation of AF-1 of RARγ is required for the activation of certain genes and for triggering primitive and visceral differentiation, while that of RXRα is required for the antiproliferative action of RA. In contrast, the induction of parietal differentiation by RA and cAMP involves RARα/RXRα pairs and phosphorylation of the RARα partner at its PKA site.

retinoid receptors were ablated by gene targeting and different combinations of specific ligands were used. By using these strategies, it has been demonstrated that RAR-RXR heterodimers are involved in the transduction of the retinoid signal, irrespective of the retinoid-induced event (cell differentiation, growth arrest or activation of RA-target genes), and that the activity of RXR is subordinated to that of its RAR partner. In WT F9 cells, most RA-induced cellular events, such as primitive and visceral differentiation, growth arrest or activation of most RA-target genes, are mediated by RARγ/RXRα heterodimers (Fig. 6 and Table 1). However, some events such as parietal endodermal differentiation involve RARα/RXRα pairs (Fig. 6 and Table 1).

The idea that a specific RAR/RXR heterodimer could be preferentially involved in mediating particular retinoid-dependent events has also been strongly supported by results of the RAR and RXR gene knockout studies performed in the mouse (Chambon, 1994; Kastner et al., 1995, 1997; Mascrez et al., 1998, and references therein). However, the interpretation of the initial results was rather equivocal since mice lacking a single RAR isotype showed a limited number of defects, owing either to functional redundancies or to the difficulty to discriminate between cell-autonomous and cell-non autonomous effects. Therefore, by assuming that there are less functional redundancies between RARs in a RXRα mutant background, the role of a particular RXRα/RAR pair in a given function was determined in compound RAR/RXRα mutants (Kastner et al., 1997; Mascrez et al., 1998). New strategies based on the creation of conditional spatio-temporally-controlled somatic mutations have been recently developed to bypass some of these problems (Li et al., 2000, and references therein). In contrast, in F9 cells which are obviously less complex than the intact animal, single gene knockouts can generate unequivocal effects in a number of instances, although they may also result in artefactual conditions, unmasking potential functional redundancies which do not occur in the wild-type situation.

A complementary strategy was developed in F9 EC cells, with the aim of establishing the role of the Activation Functions and of the phosphorylation of RARs and RXRs in RA-induced events. This strategy is based on the reexpression of deleted or mutated receptors in either $RAR\gamma^{-/-}$, $RAR\alpha^{-/-}$ or $RXR\alpha^{-/-}$ cells. Studies performed with such F9 rescue lines support several of the ideas that were previously suggested from in vitro studies, using cell-free systems or cultured transfected cells (see Table 1).

First, the RARα, RARγ and RXRα AF-2 AD core motifs contained in the LBD helix 12, are crucial for triggering endodermal differentiation and for activating most RA-target genes. This is in agreement with the capacity of this alpha amphipatic helix, an essential element of the AF-2 function, to recruit coactivators upon ligand binding, resulting in a transcriptionally competent receptor. Second, the Activating Function AF-1 also plays an important role, supporting the proposal that it could cooperate with AF-2. However, this role seems to differ from one receptor to another. For RARγ, AF-1 is crucial for the induction of certain RA-target genes and for triggering endodermal differentiation. For RARα, the result is puzzling, as this deletion mutant behaves as a dominant negative towards RARγ.

The study of RARs and RXRα rescue cell lines also provides an attractive in vivo system to study how retinoid receptor phosphorylation is involved in the mechanism of retinoid signaling (see Fig. 6 and Table 1). Phosphorylation of RARγ AF-1 domain by TFIIH appears to be necessary for the activation of certain RA-inducible genes and for triggering primitive and visceral endodermal differentiation. However, phosphorylation of the RXRα partner is not involved in the RA-induced differentiation of F9 cells, but is required for the antiproliferative action of RA. How, in RARγ-RXRα heterodimers, phosphorylation of each partner cooperates to activate the expression of RA target genes specifically involved in the differentiation and/or the growth arrest of F9 cells, remains to be seen.

That phosphorylation of RARα by PKA upon cAMP addition to RA is required for parietal differentiation, demonstrates the existence of crosstalk between the RA and the PKA pathways for parietal differentiation, through the phosphorylation of RARα at its PKA site. As the growth factor/Ras/MAPK cascade can phosphorylate in vitro the serine residues located in the AF-1 domain of RARs (Rochette-Egly et al., 1997; Bastien et al., 2000), F9 cells should provide an interesting system to determine whether hyperphosphorylation of these residues stimulates the AF-1 function and thus whether the MAPK cascade might cross-talk with the retinoid pathway, as is the case for the estrogen pathway (Kato et al., 1995; Bunone at al., 1996). Interestingly, hyperphosphorylation of RXRα by stress kinases has recently been shown to negatively modulate the activation of RA-target genes (Lee et al., 2000). Future studies are required to determine whether and how RXRα hyperphosphorylation by stress-activated kinases could suppress retinoid signaling in F9 cells.

The studies performed with WT and rescued F9 cells also show that the heterodimers which play a key role in RA-induced primitive differentiation and in the induction of expression of many RA-target genes (i.e. RARγ/RXRα heterodimers), are degraded by the ubiquitin-proteasome pathway and that this degradation depends on the phosphorylation of the RARγ partner. Such results argue that transcriptional activation and activator degradation are closely coupled events. Therefore a model has been proposed (Kopf et al., 2000), in which degradation mediated by the proteasome pathway may participate in the regulation of duration and magnitude of retinoid action. Upon ligand binding, RARγ/RXRα heterodimers bound to cognate response elements recruit coactivators, leading to activation of

transcription. A subsequent increase in ubiquitination would then result in the degradation of both heterodimeric partners. In this process, phosphorylation and ligand-dependent ubiquitination act in concert to trigger receptor degradation, thus modulating the intracellular levels of RARγ/RXRα heterodimers and controlling the magnitude of the retinoid effect.

In conclusion, through binding of cognate ligands and phosphorylation of their activation domains, retinoid receptors are highly sophisticated transducers, integrating signals belonging to several signaling pathways. Thus, F9 cells and the mutants that we have generated, provide interesting tools to study complex physiological conditions that result from the integrative cross-talks of multiple signaling pathways. As different RAR/RXR pairs have been shown to effect a given cellular event, in a cell-specific manner (Roy et al., 1995; Chen et al., 1996), the question arises as to whether there is any principle governing the role of RAR and RXR phosphorylation in physiological responses to retinoids. Studies of the consequences of RAR and RXR phosphorylation should be extended to other mammalian cell lines such as P19 EC or APL NB4 cells whose differentiation involves RXR/RAR pairs different from those operating in F9 cells (Roy et al., 1995; Chen et al., 1996). Finally, elucidating whether and how RAR and RXR phosphorylation is involved in the mouse during development and homeostasis is essential to reveal the physiological role of crosstalks between the retinoid signaling pathway and other signaling pathways that are ultimately mediated through phosphorylation of transregulators.

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