Chapter 4
Evolution of Retinoic Acid Receptors and Retinoic Acid Signaling

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Abstract Retinoic acid (RA) is a vitamin A-derived morphogen controlling important developmental processes in vertebrates, and more generally in chordates, including axial patterning and tissue formation and differentiation. In the embryo, endogenous RA levels are controlled by RA synthesizing and degrading enzymes and the RA signal is transduced by two retinoid receptors: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). Both RAR and RXR are members of the nuclear receptor superfamily of ligand-activated transcription factors and mainly act as heterodimers to activate the transcription of target genes in the presence of their ligand, all-trans RA. This signaling pathway was long thought to be a chordate innovation, however, recent findings of gene homologs involved in RA signaling in the genomes of a wide variety of non-chordate animals, including ambulacrarians (sea urchins and acorn worms) and lophotrochozoans (annelids and mollusks), challenged this traditional view and suggested that the RA signaling pathway might have a more ancient evolutionary origin than previously thought. In this chapter, we discuss the evolutionary history of the RA signaling pathway, and more particularly of the RARs, which might have experienced independent gene losses and duplications in different animal lineages. In sum, the available data reveal novel insights into the origin of the RA signaling pathway as well as into the evolutionary history of the RARs.

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Abbreviations

ADH	Alcohol dehydrogenase
AF	Activation function
BCO	β-carotene 15,15′-monooxygenase
BMP-1	Bone morphogenetic protein-1
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
CYP	Cytochrome P450
CYP26	Cytochrome P450 subfamily 26
DBD	DNA binding domain
DR	Direct repeat
FRET	Fluorescence resonance energy transfer
HPLC	High performance liquid chromatography
LBD	Ligand binding domain
RA	Retinoic acid
RALDH	Retinal dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RDH10	Retinol dehydrogenase 10
RXR	Retinoid X receptor
SDR	Short-chain dehydrogenase/reductase
USP	Ultraspiracle

Introduction

Retinoic acid (RA) is a fat-soluble morphogen derived from vitamin A that, in vertebrates, controls the organization of the anteroposterior axis and the formation and differentiation of various tissues during development. It is well known that one of the major actors of the RA signaling pathway is the retinoic acid receptor (RAR), a member of the nuclear receptor superfamily of ligand-activated transcription factors. RARs mainly act as heterodimers with the retinoid x receptors (RXRs) to activate the transcription of target genes in the presence of their ligand, all-*trans* RA (reviewed in [36]) (Fig. 4.1). In most vertebrates, including human and mouse, there are three *rar* genes (*rarα*, *rarβ* and *rarγ*) and three *rxr* genes (*rxrα*, *rxrβ* and *rxrγ*) (Fig. 4.2), each encoding several isoforms (reviewed in [51]). In fact, at the origin of vertebrates two whole genome duplications took place [4, 21, 57, 82] giving rise to the multiple RAR and RXR paralogs in vertebrates. Thus, a number of different heterodimers can be formed between these receptors and it is believed that there are more than thirty different RAR-RXR heterodimer associations, taking into account the many RAR and RXR isoforms produced, that transduce signals in the presence of all-*trans* RA [51, 59]. It is
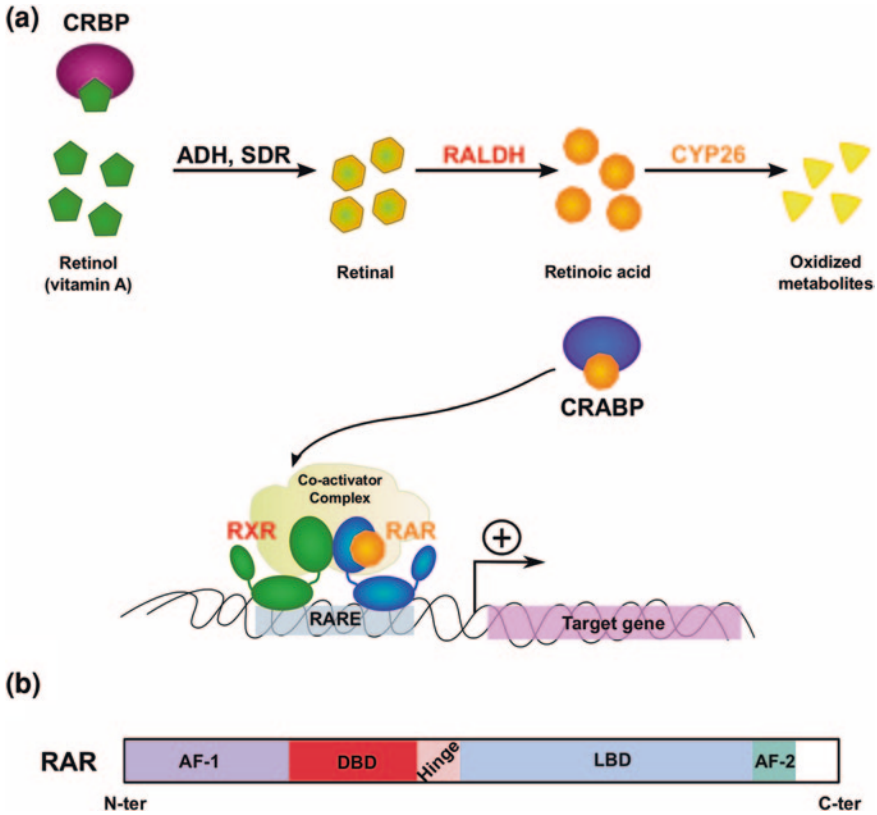
Fig. 4.1 RA signaling pathway. **a** Schematic representation of RA metabolism and signaling. Retinol is converted to RA in two steps, first retinol (vitamin A) is oxidized into retinal by a reversible reaction and then retinal is oxidized into RA. RA is transported into the nucleus where it binds to the retinoic acid receptor (RAR), which forms a heterodimer with the retinoid X receptor (RXR). Upon binding of RA, the RAR-RXR heterodimer will recruit co-activators and activate the transcription of target genes. The colors represent the evolutionary conservation: RXR and RALDH are in *red* because they are present in all metazoans and CYP26 and RAR are in *orange* because they are absent in ecdysozoans and cnidarians. ADH alcohol dehydrogenase; CRBP cellular retinol binding protein; CRABP cellular retinoic acid binding protein; CYP26 cytochrome P450 subfamily 26; RA retinoic acid; RALDH retinaldehyde dehydrogenase; RAR retinoic acid receptor; RARE retinoic acid response element; RXR retinoid X receptor; SDR short chain dehydrogenase/reductase. **b** Overview of the structure of the RAR protein: N-terminal ligand-independent transcriptional activation domain (AF-1), a centrally located DNA-binding domain (DBD) consisting of a highly conserved core region, which contains two zinc finger modules, a hinge that allows flexibility between the N- and C-terminal portion of the molecule and a C-terminal ligand binding domain (LBD), which interacts with the ligand, allowing receptor dimerization and additionally serving as a ligand-activated transcriptional activation function (AF-2) domain
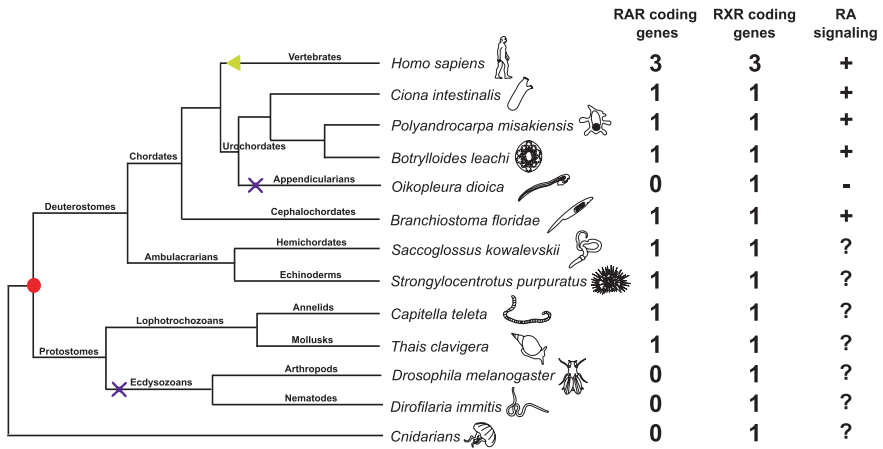
			RAR coding genes	RXR coding genes	RA signaling
Vertebrates	*Homo sapiens*		3	3	+
	Ciona intestinalis		1	1	+
	Polyandrocarpa misakiensis		1	1	+
	Botrylloides leachi		1	1	+
Appendicularians	*Oikopleura dioica*		0	1	-
Cephalochordates	*Branchiostoma floridae*		1	1	+
Hemichordates	*Saccoglossus kowalevskii*		1	1	?
Echinoderms	*Strongylocentrotus purpuratus*		1	1	?
Annelids	*Capitella teleta*		1	1	?
Mollusks	*Thais clavigera*		1	1	?
Arthropods	*Drosophila melanogaster*		0	1	?
Nematodes	*Dirofilaria immitis*		0	1	?
	Cnidarians		0	1	?

Fig. 4.2 Evolution of RAR. Schematic phylogenetic distribution of RARs and RXRs in bilaterian animals: RARs are generally present in chordates (vertebrates, tunicates and cephalochordates) and have recently been identified in the genomes of ambulacrarians (hemichordates and echinoderms) and lophotrochozoans (annelids and mollusks). In general, RARs seem to be absent from ecdysozoan genomes. RAR might thus have already been present in the last common ancestor of deuterostomes and protostomes, called Urbilateria [12]. Given the absence from ecdysozoan genomes, RAR might have secondarily been lost in this lineage. Moreover, RAR has probably also been secondarily lost in a tunicate lineage, the appendicularians. In vertebrates, the *rar* genes have been duplicated, with, for example, *Homo sapiens* possessing three *rar* paralogs (*rarα*, *rarβ* and *rarγ*). In contrast, RXRs are present in all metazoan lineages. In addition, *rxr* genes have also been duplicated in vertebrates as, for example, *Homo sapiens* possessing three *rxr* paralogs (*rxrα*, *rxrβ* and *rxrγ*). The presence of a functional retinoic acid (RA) signaling pathway is indicated by a + or − sign, however, in many lineages this has not been determined (?). Numbers indicate the number of RAR and RXR coding genes in a given lineage. The *red dot* indicates the probable origin of rar and the *yellow triangle* indicates the whole genome duplication that took place in the vertebrate lineage and the purple *x* indicates the loss of RAR. Protein accession numbers: (*source* http://www.ncbi.nlm.nih.gov/, excepting the *Capitella teleta* sequence, which is accessible at: http://genome.jgi-psf.org/Capca1/Capca1.home.html): *Homo sapiens* RARα (P10276), RARβ (P10826), RARγ (P13631), RXRα (P19793.1), RXRβ (P28702.2) and RXRγ (P48443.1), *Ciona intestinalis* RAR (NP_001072037) and RXR (NP_001071809), *Polyandrocarpa misakiensis* RAR (BAA25569) and RXR (BAA82618), *Botrylloides leachi* RAR (DQ523226) and RXR, *Branchiostoma floridae* RAR (AAM46149) and RXR (AAM46151), *Saccoglossus kowalevskii* RAR (XP_002742241) and RXR (ADB22634), *Strongylocentrotus purpuratus* RAR (XP_779976) and RXR (XP_784246), *Capitella teleta* RAR (168520) and RXR (186691), *Thais* (*Reisha*) *clavigera* RAR (BAN82614.1) and RXR (E9RHD8), *Drosophila melanogaster* USP (AAF45707), *Dirofilaria immitis* USP (AAM08269) and *Tripedaliacystophora* RXR (AAC80008)

also important to mention that RXR is believed to be a receptor for a specific RA isomer, 9-*cis* RA, although the in vivo relevance of this observation is still debated ([11, 47, 68] and references therein). Moreover, other ligands such as docohexaenoic acid or phytanic acid, among others, have been proposed as ligands for RXR [22, 60]. The functional role of RXR in the heterodimer is outside the scope of

this chapter, but it is nonetheless important to point out that RXR is not a passive partner, and the true nature of its in vivo role remains to be described (see [32] and references therein).

History: Production, Metabolism and Signaling by Retinoids in Vertebrates

The Structure of the RAR-RXR Heterodimer

Like other members of the nuclear receptor superfamily, RARs are modular proteins, possessing several domains, which carry out specific functions required for their activities as ligand-regulated transcription factors (Fig. 4.1). The RAR-RXR heterodimer binds to DNA on specific sequences called retinoic acid response elements (RAREs), which, most frequently, consist of direct repeats (DRs) [2, 15] (Fig. 4.1).

In the apo form, which corresponds to the receptor without the ligand, RAR, together with RXR, interacts with transcriptional co-repressors, such as NCoR or SMRT, and represses the transcription of target genes (reviewed in [36]). Following ligand binding, the LBD undergoes a conformational change during which the most C-terminal helix (H12) forms a lid that closes the hydrophobic pocket in which the ligand is buried (reviewed in [36]). It has been shown that this conformational change significantly alters the composition of the proteins that interact with the LBD. The conformational change causes dissociation of the co-repressors and the binding of co-activators, such as the members of the p160 proteins (SRC1, 2 and 3) that induce histone acetylation and subsequently, transcriptional activation (reviewed in [66]. This is, however, an oversimplified presentation of RAR-RXR functions, as many different types of co-repressors, co-activators and chromatin remodeling complexes work together to orchestrate the remodeling of chromatin linked to transcriptional regulation [3, 34]. Recently, older models of unliganded RAR action have been challenged by new genome-wide analyses of RAR target genes (see Chaps. 9 and 10). It has become clear that ligand binding generally increases the ability of RAR-RXR to interact with the regulatory regions of at least some target genes [10, 67, 69].

Regulation of Endogenous RA Levels

Many studies have thoroughly characterized the function of RAR in vertebrates, focusing mainly on developmental and physiological aspects [35, 86]. Other studies have characterized the biochemical pathways that produce RA in vivo. These data have revealed that the RA precursor, retinol (which is vitamin A), is catabolized to

retinal in a reversible manner by alcohol dehydrogenase (ADH) [8, 54, 103] or the short-chain dehydrogenase/reductase (SDR) enzymes, such as RDH10 [102]. This reaction is followed by an irreversible oxidation step of retinal to RA by retinal dehydrogenases (RALDHs), which is the rate-limiting step of this pathway [25]. There are three RALDH enzymes in most vertebrates, and RALDH2 is the most relevant RALDH during embryonic development, with its expression allowing us to infer, at least grossly, the regions of the embryo characterized by high endogenous RA levels (see [78]). Importantly, this zone of high endogenous RA levels gives rise to two RA gradients, one oriented anteriorly and one posteriorly in the embryo, as has recently been revealed in developing zebrafish using a visualization technique based on fluorescence resonance energy transfer (FRET) [91].

Within a target cell, retinol is associated with cellular retinol binding protein (CRBP), and RA is associated with the cellular retinoic acid binding protein (CRABP) [72]. Cellular and tissue concentrations of RA are generally regulated through homeostatic processes involving both RA production and degradation, with enzymes of the cytochrome P450 subfamily 26 (CYP26) principally mediating the catalysis of RA to inactive products like 4-oxo RA and 4-hydroxy RA [76]. Three genes encoding CYP26 enzymes (CYP26A1, CYP26B1 and CYP26C1) exist in vertebrates [99]. Moreover, in vitro experiments have identified further RA-degrading CYP enzymes that may also be involved in the RA catalysis process, such as CYP1A2, 2A4, 2A6, 1B1, 2B1, 2B6, 2C3, 2C7, 2C8, 2C9, 2D6, 2E1, 2E2, 2G1, 3A4/5, 3A6, 3A7, and 4A11. However, the in vivo functions of these enzymes remain unclear [37, 61, 81].

Development of the Field: Evolutionary Origins of RA Signaling

In contrast to the situation in vertebrates, where the roles and mode of action of RA are relatively well understood, very little is known about the origins of the RA signaling pathway. Only a few studies have focused on the functions of RAR in non-vertebrate animals. Among non-vertebrates, only representative RAR receptors found in other chordates, i.e. in tunicates and cephalochordates, have been functionally characterized [27, 28, 30, 31]. However, the recent identification of some components of the RA machinery in the genomes of non-chordate animals has provided some useful hints that shed new light on the action of RA and the presence of a RA signaling pathway in metazoan animals. For example, homologs of *rar*, *cyp26* and *raldh* have been identified in the genomes of ambulacrarians (sea urchins and acorn worms) and lophotrochozoans (annelids and mollusks) (Fig. 4.2) [1, 12, 13, 65, 92]. Our current understanding of the origin of RAR suggests the evolutionary history of RAR was influenced by specific losses and duplications in particular animal lineages. Altogether, this analysis allows us to conclude that, evolutionarily speaking, the RA signaling pathway is more ancient than originally thought.

Current State of the Field: RA Signaling in Non-vertebrate Animals

Presence or Absence of RA-Signaling in Metazoan Genomes: Chordates

The RA signaling pathway was long thought to be vertebrate-specific. However, it is now well established that most of the components of the RA machinery are present and functional in all chordate phyla. More particularly, single *rar* homologs have been identified and functionally characterized in the following chordates (Fig. 4.2): *Ciona intestinalis* [31], *Botrylloides leachi* [80] and *Polyandrocarpa misakiensis* [40] (all of which are ascidian tunicates), as well as in the cephalochordate *Branchiostoma floridae* also referred to as amphioxus [28].

As previously mentioned, vertebrates possess at least three RARs, RARα, RARβ and RARγ (Fig. 4.2), but as indicated, only one RAR is present in chordates (cephalochordates and ascidian tunicates) (Fig. 4.2). The evolution of the chordate RA signaling pathway has been studied extensively [27, 30, 31, 88]. In amphioxus it has been shown that RAR and RXR are able to heterodimerize, bind RA and activate the transcription of genes upon RA binding [28]. Moreover, it was shown that the amphioxus RAR has a ligand-binding pocket structure similar to RARβ [27]. RA functions have been described in the embryonic development of amphioxus. For example, it has been shown that RA regulates the anteroposterior patterning of the CNS and neuronal specification in a *hox*-dependent manner [88]. RA also mediates the patterning of the ectoderm and the formation of the tail fin in this specie [14]. In addition, orthologs of *cyp26*, *rdh* and *raldh* have been identified in the genome of amphioxus, however, their function remains to be assessed. In contrast, there is no evidence of the existence of genes coding for proteins responsible for retinol storage, transport and cellular uptake, suggesting that these might have appeared specifically in the vertebrate lineage [14].

Components of the RA machinery have been identified in tunicates. It was shown that RARs are present in *Ciona intestinalis* [31], *Botrylloides leachi* [80] and *Polyandrocarpa misakiensis* [40] that bind and are activated by RA. However, the function of the enzymes involved in RA synthesis and metabolism has not been assessed yet in these animals [14]. In addition, RA signaling has been secondarily modified in different lineages [14]. For example, there is a partial loss of the RA-dependent regulation of the *hox* code in ascidians [14], but the appendicularian tunicate, *Oikopleura dioica*, stands out as an exception because it lacks the genes coding for the main actors of the RA signaling pathway, such as, *rar*, *aldh1* and *cyp26* [13]. In the course of evolution, these genes were very likely secondarily lost in this animal since these gene are present in all the other urochordate species studied, such as *Ciona intestinalis* [31], *Botrylloides leachi* [80] and *Polyandrocarpa misakiensis* [40]. Of interest, it seems that the anterioposterior patterning of *O. dioica* during embryonic development may be RA-independent, although detailed characterization of the developmental pathways used by this

species is still lacking. These observations suggest that anteroposterior patterning can be achieved using many different signaling pathways and is not necessarily dependent on RA.

In conclusion, the RA signaling pathway is present and functional in chordates, however, it should be noted that, compared to the situation in vertebrates, very little is known about the regulation of this signaling cascade and its function in invertebrate-chordates.

Presence or Absence of RA-Signaling in Metazoan Genomes: Non-chordates

Bioinformatic analyses have revealed that genes involved in the RA signaling machinery are present in the genomes of a variety of non-chordates (ambulacrarians and lophotrochozoans) [1, 12, 13, 65, 92]. Based on phylogenetic analysis of the nuclear hormone receptor superfamily, it was hypothesized that a proto-RAR might have been present in Urbilateria, the last common ancestor of all bilaterians [4].

Homologous genes of *rar* have so far been identified in the genomes of the following non-chordate invertebrates (Fig. 4.2): the ambulacrarians *Saccoglossus kowalevskii* (a hermichordate) and *Strongylocentrotus purpuratus* (an echinoderm) [13, 65] and the lophotrochozoans *Lottia gigantea* (a mollusk) and *Capitella teleta* (an annelid, formerly *Capitella capitata*) [12]. The phylogenetic analyses performed and the surprisingly high degree of amino acid conservation of the RARs found in these species indicate that these receptors may be *bona-fide* RARs that might be able to bind RA [12]. These findings allow us to conclude that the gene encoding RAR has a much more ancient evolutionary origin than previously believed. In particular, the fact that a clear rar ortholog is found in lophotrochozoans (annelids and mollusks) strongly suggests that a *rar* gene was already present in the ancestor of all bilaterians. This has important implications for the evolution and diversification of metazoan animals, since it indicates that functional roles for RA in developmental patterning might be evolutionarily much more ancient than originally thought (reviewed in [12]). However, recently, the characterization of a RAR from the mollusk *Thais clavigera* showed that this receptor is unable to bind RA [97], raising the question of the function of RAR and RA in non-chordate animals. Thus, the unambiguous experimental demonstration of a functional RAR and of a functional RA signaling cascade outside the chordate phylum still remains elusive.

In contrast, previous in silico analyses did not identify *rar* homologs in appendicularian tunicates (see above), ecdysozoans (arthropods and nematodes), and cnidarians (Fig. 4.2). These results allow two tentative conclusions: (1) if the absence of *rar* from cnidarian genomes is confirmed, for example by the analysis of other genomes, the origin of RAR and RA signaling can probably firmly be placed at the base of bilaterian animals and not at the base of metazoan animals. Given that anteroposterior patterning is an important feature of bilaterians, it is tempting to speculate that RAR might have played a role in the emergence of

bilaterian body axes and, particularly, in the evolution of anteroposterior patterning systems, but this remains speculative given the very limited amount of data currently available on the role of RA outside chordates (see below); (2) the absence of *rar* in appendicularian tunicates and ecdysozoans suggests that these genes have been independently lost in these animal lineages and indicate that a complex anteroposterior patterning system can be established in the absence of RAR.

Homologs of the enzymes involved in RA metabolism, such as RALDH and CYP26 have also been identified in non-chordate animals. For example, *raldh* homologs have been identified in the genome of ambulacrarians (*Strongylocentrotus purpuratus* and *Saccoglossus kowalevskii*), lophotrochozoans (*Lottia gigantea* and *Capitella teleta*), ecdyzosoans (*Caenorhabditis elegans*, *Daphnia pulex* and *Drosophila melanogaster*) and cnidarian (*Nematostella vectensis*) [1, 12, 13, 65, 92]. In contrast, *cyp26* homologs have only been identified in lophotrochozoans and ambulacrarians [1, 12, 13, 65, 92]. Altogether, these data reinforce the notion that the RA signaling pathway might have been present in Urbilateria, the last common ancestor of protostomes and deuterostomes. However, the unambiguous experimental demonstration of a functional RAR and of a functional RA signaling cascade outside the chordate phylum still remains elusive.

In contrast, *rxr* homologs have been identified in the genomes of various non-chordate phyla including lophotrochozoans [9], ecdysozoans [104] and basal metazoan species, such as cnidarians [29, 55] and sponges [100]. Therefore, RXR seems to have a more ancient evolutionary origin than RAR. Apparently, the RXR receptors have also experienced a complex evolutionary history. For example, in vertebrates, several paralogs of RXR were acquired following whole genome duplication events [77] and in insects, ligand recognition by USP (i.e. RXR) has been altered independently in different lineages [16, 44, 46]. For instance, it was shown that the USP from *Tribolium castaneum* does not have a ligand-binding pocket and, thus, does not bind and is not activated by RXR ligands despite the high degree of identity between these receptors [46]. In addition, in mecopteridan USPs, the ligand-binding pocket is very large and is occupied by a phospholipid [6, 17]. These data reveal the evolutionary plasticity of the ligand binding pocket of nuclear hormone receptors and highlight the requirement for experimental analyses of divergent receptors.

RA Derivatives in Non-chordate Invertebrates

In vertebrates, RAR is able to bind at least two different isomers of RA, all-*trans* RA and 9-*cis* RA, whereas RXR only binds to 9-*cis* RA in vitro. Moreover, many studies have shown that treatment of vertebrate species with all-*trans* RA or 9-*cis* RA causes developmental defects and malformations [79]. In an effort to better understand the biological roles of each RA isomer, efforts have been made to measure the presence of these compounds in different organs of vertebrates. The presence of all-*trans* RA during development was detected, for example, in mouse, rat and human serum,

liver, kidney, brain and testis [39, 48, 87]. The concentration of all-*trans* RA in these tissues was between 7 and 10 nM, however, it was lower in the serum [48].

In contrast, far less is known about the metabolism of 9-*cis* RA and the presence of 9-*cis* RA in vivo. Until recently, the biological significance of this isomer remained controversial. In fact, 9-*cis* RA was first detected by high performance liquid chromatography (HPLC) in mouse liver and kidney [39] and rat epididymal tissue [75] at concentrations two or three times lower than those of all-*trans* RA. Subsequent analytical assays, however, failed to detect this isomer in liver, kidney and other murine tissues (reviewed in [47]). This failure to detect 9-*cis* RA in vivo has contributed to the controversy about the biological relevance of this RA isomer. In contrast, by using liquid chromatography/tandem mass spectrometry a recent study has revealed the presence of 9-*cis* RA in mouse pancreas [49]. This work further established that 9-*cis* RA is able to attenuate glucose-stimulated insulin secretion in the pancreas [49]. These data not only show that 9-*cis* RA is a naturally-occurring RA isomer in the pancreas, but also that 9-*cis* RA is biologically active. In fact, it is possible, and even likely, that 9-*cis* RA can simply not be detected in other tissues, because its levels are below the limit of detection of the employed analysis methods.

In addition to mice, rats and humans, endogenous RA levels have also been measured in a number of other animals. But before reviewing these data, it has to be mentioned that derivatives of retinol, generally termed retinoids, are also involved in the visual cycle and that in particular 11-*cis* retinaldehyde plays a crucial role in vision. This aspect is not considered in the following discussion, but it is nonetheless important to be aware of the retinoid derivatives that participate in vision as it helps to explain the wide distribution in metazoan animals of various enzymes able to act on retinoid compounds. For example, 9-*cis* RA was detected in the regenerating limb of urodele amphibians [98], all-*trans* RA was detected in budding tunicates [52] and both all-*trans* RA and 9-*cis* RA were detected in the chordate amphioxus [20].

In non-chordates, endogenous all-*trans* RA and 9-*cis* RA were detected by HPLC in the central nervous system and the hemolymph of the mollusk *Lymnea stagnalis* [23]. All-*trans* RA concentrations were higher than those of 9-*cis* RA with all-*trans* RA concentrations estimated at 693 nM in the central nervous system and 155 nM in the hemolymph, whereas, for 9-*cis* RA, the estimated concentrations were 380 nM in the central nervous system and 120 nM in the hemolymph [23]. These values are thus relatively high, when compared to the values typically reported in mammals, but the significance of this difference is still unclear. Similarly, in the locust embryo, both all-*trans* RA and 9-*cis* RA were detected [74]. The all-*trans* RA concentration in the whole embryo was estimated to be 3 nM and the 9-*cis* RA concentration in the locust embryo was around 1.5 nM [74]. All-*trans* RA and 9-*cis* RA were also detected in limb blastemas of the fiddler crab during regeneration. Here, all-*trans* RA concentrations were 19 pg/μg protein/blastema and 9-*cis* RA concentrations were 83 pg/μg protein/blastema [41].

There are other RA isomers, such as 9,13-di-*cis* RA and 13-*cis* RA, that, at least in vertebrates, are characterized by reduced or absent biological activity [43, 95]. Interestingly, these RA isomers are distributed relatively widely in

different mammalian tissues, for example, serum, brain, liver, kidney, adipose tissue, muscle, spleen and testis [50]. Moreover, they have also been detected in invertebrates, including sponges, which are basal metazoans. Sponges contain both retinyl esters and 13-*cis* RA and are able to accumulate β-carotene [5, 71]. In addition, retinol and retinyl esters have been detected in mollusks, which also possess some kind of capacity to endogenously store retinoids [33].

In nematodes, the only evidence of a possible action of RA has been reported in the parasitic nematodes *Brugia malayi* and *Onchocerca volvulus* [94, 101]. In both worm species only retinol could be detected [94, 101] and it was shown that *Brugia malayi* worms were able to take up and accumulate all-*trans* RA in their tissues [101]. In addition, it was shown that the development of the parasitic nematode *Litomosoides carinii* in vitamin A-deficient cotton rats was delayed [93]. Together, these data suggest that retinoids may play a role in the development of parasitic nematodes, however, it is important to stress that there is no RAR in nematodes and that a homolog of USP (i.e. RXR) has only been identified in a single species of parasitic nematodes [90].

Role of RA Derivatives in Non-chordate Invertebrates

As discussed above, in vertebrates, both RA isomers, all-*trans* RA and 9-*cis* RA, are not only present, but also biologically active. In fact, it is known that RA controls many developmental processes of vertebrates [79]. Indeed, exposure of developing embryos to exogenous RA causes severe defects in vertebrates and more generally in chordates. However, outside chordates, evidence for biological functions of RA is scarce and only a few studies have addressed the impact of RA on embryonic development. For example, in many mollusk species, such as, *Lymnaea stagnalis*, *Physa fontinalis* and *Bithynia tentaculata*, constant treatment starting at gastrulation with all-*trans* RA (100 nM) affects eye formation [19]. In addition, the use of higher concentrations of all-*trans* RA (1 μM) on *Lymnaea stagnalis* embryos causes various malformations, for example, eye and shell defects and, at times, growth arrest [19]. Moreover, it was shown that RA treatments were only effective during gastrulation as treatment of embryos after gastrulation did not cause any evident developmental defects [19]. This suggests that RA might control specific developmental events in this species, which is reminiscent of the role played by RA during vertebrate development.

Treatment of cultured neurons from the mollusk *Lymnaea stagnalis* with all-*trans* RA (100 nM) induces neurite outgrowth and growth cone turning [23, 24]. A comparable effect was observed in vertebrates with a similar RA concentrations [18]. 9-*cis* RA treatments also have an effect on mollusks. For example, in the mollusk *Thais clavigera*, the injection of 9-*cis* RA (1 μM) causes imposex, i.e. the development of male organs in females [73]. Taken together, although the available evidence is still very fragmentary and based mainly on morphological observations, these data on effects of RA treatments in mollusks are nonetheless indicative of possible functional roles of RA derivatives in mollusks. However,

recently a RAR from the mollusk *Thais clavigera* has been characterized and it was shown that this receptor is unable to bind RA [97], thus suggesting that effects of RA on mollusks might be RAR independent.

Data are also available that describe the effects of treatments with RA derivatives in echinoderms. For example, treatment of micromere-derived cells from the sea urchin *Hemicentrotus pulcherrimus* with all-*trans* RA (100 nM) induces pseudopodial cable growth [56]. Intriguingly, no effect was observed after treatment of embryos of the sea urchin *Paracentrotus lividus* with all-*trans* RA (20 µM). The embryos seem to develop normally, although their development is delayed [89].

Finally, there is growing evidence for a role of RA derivatives in tissue formation and regeneration of a wide variety of animals, including platyhelminthes [83], insects [38], crustaceans [42], cnidarians [70] and sponges [45]. This role might be related to the known effect of retinoids in controlling fin regeneration in zebrafish [7] and limb regeneration in urodeles [98]. In addition, retinoids appear to be implicated in regulating tissue regeneration in the fruit fly *Drosophila melanogaster* [38]. Mutations in the genes coding for enzymes responsible of retinoid metabolism, such as BCO (β-carotene 15,15′-monooxygenase) and a retinaldehyde dehydrogenase, generated a delay in tissue regeneration in this species. A similar effect was observed when flies were deprived of caroteinoids, a natural source of retinoids, and the normal timing was restored by supplementation of retinoids [38]. These results are very surprising because in the fruit fly retinoids were thought to be involved exclusively in vision. Moreover, there is no RAR in the fruit fly genome and USP (i.e. RXR) is unable to bind retinoids (see [46] and references therein).

Moreover, in the platyhelminthe *Girardia tigrina*, treatment with exogenous all-*trans* RA (0.5 mM) disrupts anterior but not posterior regeneration [83]. In the fiddler crab *Uca pugilator*, treatment of regenerating limb buds with high concentrations of all-*trans* RA (50 µM) causes growth delay and malformations of the limb bud [42]. In the cnidarian *Hydractinia echinata*, treatments with retinol, retinaldehyde and all-*trans* RA (1 nM to 1 µM each) increase the regeneration rate, number of tentacles and budding rate [70]. Sponges are also affected by treatment with all-*trans* RA (50 µM), which causes morphogenetic malformations of buds and gemmules [45]. In addition, it was shown that treatment with all-*trans* RA (1 mM) upregulates the expression of the gene encoding the BMP-1 (bone morphogenetic protein-1) homolog in the sponge *Suberites domuncula* [71] and downregulates the expression of the *c-myb* gene in the sponge *Geodia cydonium* [5]. Altogether, these observations suggest that RA might have various pleiotropic effects in metazoan animals that may range from organ formation to regeneration.

Relevance

It is important to point out that from these experiments we cannot formally conclude that endogenous retinoids play physiological roles in these different animals. In fact, some of the effects observed upon RA exposure might be caused

exclusively by the toxicity of the molecule at high dose or by its transformation into a different active compound. In addition, even if, in a given animal, the molecule acts on a specific target at a relatively weak concentration, one cannot necessarily conclude that the relevant metabolic pathway exists endogenously in that animal [62]. The case of bisphenol A is exemplary for this situation: bisphenol A is an artificially produced molecule that has been shown to exert very strong hormonal effects in mammals by regulating the activity of estrogen receptors [84]. Although these effects inadvertently exist in vivo, bisphenol A is certainly not an endogenously-produced hormone.

Coming back to RA, its derivatives and their potential biological functions, the solution of the problem seems rather straightforward: the receptors need to be characterized in full, including both molecular capacities and physiological functions, and the data hence obtained need to be correlated with the in vivo retinoid content of a given species. Of course, this work approach will not yield insights into retinoid functions that are not mediated by RAR and/or RXR. Even so, to date we completely lack a functional characterization of non-chordate RARs and we have thus no indication whatsoever of the possible ligand of these RARs. It could be all-*trans* RA, another RA isomer or some other compound of unknown nature. Therefore, we cannot conclude that the effects observed upon RA treatment are indeed RAR-dependent or even RA signaling-dependent.

Future Directions

With the sequencing of the genomes of many different animal species, it has become evident that the RA signaling pathway, that was initially thought to be chordate specific, has a much wider phylogenetic distribution than originally thought. In fact, some of the components of this pathway are found in the genomes of a wide variety of non-chordate taxa. For example, many components of this signaling pathway, such as the homologs of *rar*, *raldh* and *cyp26* have been identified in the genomes of ambulacrarians and lophotrochozoans [1, 12, 13, 65, 92]. Although an experimental validation of the biological functions of these receptors and metabolic enzymes is still lacking, these data nonetheless suggests that some kind of RA signaling pathway was probably already present in the last common ancestor of all bilaterian animals: Urbilateria [12].

Recent data have highlighted that RAR, in addition to being a classical nuclear receptor regulated by the binding of its ligand is also a protein whose activity can be controlled by phosphorylation [86]. Several studies have shown that these phosphorylation events are important for the full regulatory potential of RARs. It is still unclear how this level of regulation evolved but a recent phylogenetic analysis has shown that some, but not all, of those sites, are conserved between RARs from various taxa, including vertebrates and invertebrates [85]. This is, in particular, the case of the serine phosphorylation site located in the N-terminal A/B region of the receptor that is present in the RARs of vertebrates, amphioxus and ascidian tunicates (such as *Ciona intestinalis*) and that may also be present in protostome

RARs. It will therefore be of great interest to study how the regulatory logic controlling these invertebrate receptors has been set up. Similarly, recent work has revealed the evolutionary conservation in vertebrates of RAR targets through the conservation of direct repeats DR5, DR2 and related elements in target gene promoter regions [58]. Again, almost nothing is known about RAR target genes outside of vertebrates, but it can be anticipated that studies in non-vertebrates will yield exciting insights into the evolutionary diversification of the gene regulatory network controlled by RA and its receptors.

The RA signaling pathway has evolved differently in chordates, lophotrochozoans, ambulacrarians and ecdysozoans. The receptor controlling this pathway in vertebrates, RAR, is present in all these groups except in ecdysozoans where it seems to have been lost. However, it should be noted that, given the limited number of species studied and the intriguing biological effects of RA derivatives in insects and crustaceans, one should not conclude that this pathway was lost altogether in ecdysozoans. Moreover, although lophotrochozoans and ambulacrarians posses an evident *rar* homolog, there is still no experimental evidence showing that these genes encode *bona-fide* RARs that are capable of binding RA and of activating transcription in a ligand-dependent fashion.

Thus, it is important to be cautious and to remember that the presence of the ortholog of a given vertebrate gene in a distant organism does not necessarily imply the presence of the identical function known for the product of this gene in vertebrates [62]. For example, an ortholog of the estrogen receptor is present in annelids and mollusks, but several studies have shown that the mollusk orthologs do not bind estrogens [96], whereas the annelid otholog are able to bind estrogens at high concentrations [53]. In addition, it seems that estrogens might not even be present endogenously in these species [26, 63, 64]. Therefore, future research should focus on a detailed characterization of the functions of the components of the RA signaling pathway in a wide variety of different metazoan species.

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