

## Retinobenzoic Acids and Nuclear Retinoic Acid Receptors

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**ABSTRACT.** Retinoids (retinoic acid and its analogs) are widely involved in the control of cell proliferation, cell differentiation and embryonic development. A series of potent and novel synthetic retinoids named retinobenzoic acids has been developed. Retinobenzoic acids have proven to be useful tools in the investigation of the molecular mechanisms of retinoidal actions. Retinoids elicit their biological effects by binding to and activating specific nuclear receptors for retinoids (RAR's) which belong to the steroid/thyroid nuclear receptor superfamily. Recent investigations concerning the structure and function of nuclear receptors, namely of RAR's, are reviewed. Three subtypes of RAR (RAR- $\alpha$ , RAR- $\beta$  and RAR- $\gamma$ ) have been identified so far. Each RAR is considered to play particular roles in the retinoidal actions. The role of each RAR is discussed in relation to their features and to the structure-activity relationships of retinoids. RAR's act as retinoid-dependent transcription factors which bind to a specific site of the gene and control its expression. Recent progress in the investigation of the interaction of RAR's with the responsive genes and with nuclear co-factors is reviewed. The diversity of retinoidal actions are possibly explained by the diversity of RAR's in their structure and in their spatial and temporal distribution, by the diversity of base sequences which interact with RAR's, by the diversity of cell type-specifically determined hierarchy of gene expression, and by the diversity of the nuclear factors which interact with RAR's.

Vitamin A was initially recognized in 1915 as a fat soluble nutrient which is necessary for normal growth of mammals. Later, the structure of the nutrient was identified as retinol, and its metabolic oxidation was established. Retinol is reversibly oxidized to retinal *in vivo*. Retinal is further oxidized to retinoic acid (RA) through critically regulated and irreversible metabolic pathways. Though the function of retinal in vision and the function of retinol (or maybe of its unknown metabolites) in spermatogenesis were established, the major effects of vitamin A in keeping normal growth and life are considered to be exerted by RA.

The effects of RA to modulate the growth and differentiation of a wide variety of normal and transformed cells were established. RA has also proved to be widely involved in the control of embryonic development and cell differentiation. In addition, RA has been known to modify pattern formation in vertebrates. Thus, RA has a fundamental and essential role in various life processes. Such biological effects elicited by RA are called

retinoidal actions.

From the clinical standpoint, RA has attracted attention because the compound has been found to be useful in the treatments of vitamin A-deficiency, proliferative dermatological diseases including skin cancer, leukemia and some types of tumor, and in chemoprevention of cancer. Under such circumstances, a huge number of RA analogs have been synthesized for the purpose of clinical application. Such compounds which possess retinoidal activities including RA are referred to as retinoids in this article.

Through investigations on the biological effects of retinoids, it was recognized that the mechanism of retinoidal action is very similar to that of steroid and thyroid hormones. The molecular mechanism of actions of steroid and thyroid hormones has been established to involve the regulation of specific gene expression through binding to and activating the specific nuclear receptors. The recognition of the similarity between the mechanism of retinoidal action and that of steroid and thyroid hormones resulted in the definition of retinoids as the compounds which elicit specific biological responses (retinoidal actions) by binding to and activating a specific receptor or set of receptors. Several investigators were prompted to identify the nuclear receptors for retinoids. At the end of the 1980's, the author and co-workers identified human nuclear retinoid receptors by

Abbreviations: RAR, retinoic acid receptor; RA, retinoic acid; Am80, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)-benzoic acid; Ch55, (E)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl] benzoic acid; TR, thyroid hormone receptor; RARE, retinoic acid response element; TRE, thyroid response element; HPLC, high performance liquid chromatography; CRABP, cellular retinoic acid binding protein.

the use of novel synthetic retinoids. The three genes encoding the receptors (RAR- $\alpha$ , RAR- $\beta$  and RAR- $\gamma$ ) were independently cloned by some groups of investigators in France, the United States and Japan.

In this article, the author reviews the development of synthetic retinoids and the molecular mechanism of retinoidal action in relation to the structure-activity relationships of retinoids and to the properties of established retinoid receptors (RAR's).

**I. Retinobenzoic acids.** A number of compounds which show retinoidal activities have been reported. Some compounds, *i.e.*, etretinate and arotinoids, whose chemical structures are apparently superimposable on that of RA, have been found to be potent retinoids and could be used clinically. However, these compounds, as well as RA, possess the clinical disadvantages of high toxicity (hypervitaminosis A) and teratogenicity. Their toxicity and teratogenicity are long-lasting because the compounds are highly lipophilic and difficult to eliminate from the body. It is desirable to develop novel retinoids that are more specific than conventional retinoids and can be eliminated promptly from the body. Such compounds are expected to be developed by the introduction of hetero-atoms like nitrogen and/or oxygen atoms into the molecular skeleton. The introduced hetero-atoms are considered to confer polarity on the molecule.

Under such conditions, a series of novel retinoids called retinobenzoic acids whose generic structure is represented in Fig. 1 has been developed (1). The structure-activity relationships of retinobenzoic acids have been well documented. Briefly, retinobenzoic acids are constituted of a *para*-substituted benzoic acid moiety and a phenyl ring moiety substituted with hydrophobic alkyl group(s). These two aromatic rings are connected with the group X (Fig. 1) which can be NHCO (amide), COCH=CH ( $\alpha$ ,  $\beta$ -unsaturated carbonyl), COCH<sub>2</sub>CO (1,3-dicarbonyl), N=N (azo), OCO (ester), and so on. The group X plays a role in locating the two aromatic

moieties at spatially suitable positions with respect to each other for the binding to the receptor. The bulkiness and position of the group R are critical for the potency of retinoidal activity elicited by the retinobenzoic acids. The group R should be a medium size alkyl group (*ex.* isopropyl) positioned at *meta* (R<sub>2</sub>) or *para* (R<sub>3</sub>). The structure-activity relationships concerning the group R's are common for all of the series of retinobenzoic acids bearing various group X's, suggesting that all of the retinobenzoic acids elicit their retinoidal activities by binding to a common receptor(s).

Among a huge number of retinobenzoic acids, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl-carbamoyl)-benzoic acid (Am80) and (E)-4-[3-(3,5-di-*tert*-butylphenyl)-3-oxo-1-propenyl]-benzoic acid (Ch55) possess quite potent retinoidal activities and unique features. The structures of Am80, Ch55 and RA are shown in Fig. 2 as well as the ED<sub>50</sub> value in the cell differentiation induction assay using human promyelocytic leukemia cells HL-60. These retinobenzoic acids, Am80 and Ch55, which have stronger retinoidal activities than RA, are expected to bind to a specific retinoic acid receptor (RAR) with high affinity.

Am80 and Ch55 possess potent retinoidal activities in a number of biological assay systems including growth promotion of vitamin A-deficient mammals, regulation of cell proliferation and differentiation, regulation of specific gene expression, morphogenesis of vertebrates, and so on. In such biological assay systems, Am80 and Ch55 elicit the same effects with those elicited by RA. One feature, or only one so far recognized, of Am80 and Ch55 which is different from that of RA is the lack of binding affinity for the cellular retinoic acid binding protein (CRABP). CRABP exists in the cytosolic fraction of many tissues and cultured cells. The lack of binding affinity of Am80 and Ch55 for CRABP indicates that CRABP is not essential for retinoidal actions.

The identification of RAR(s) in terms of specific binding activity for RA was complicated by the existence of large amounts of CRABP in many tissues or cells. By

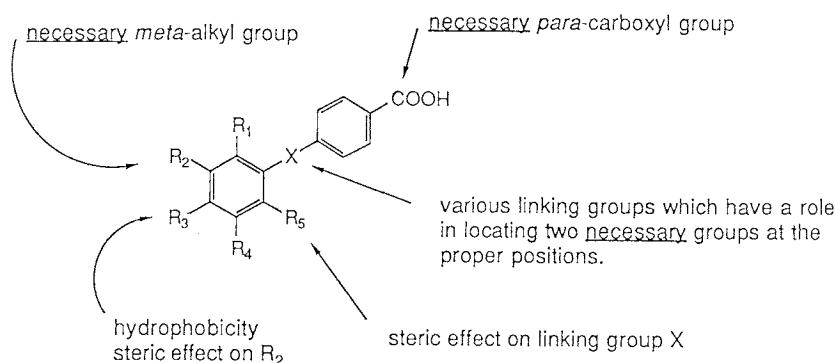


Fig. 1. The generic structure of retinobenzoic acids.

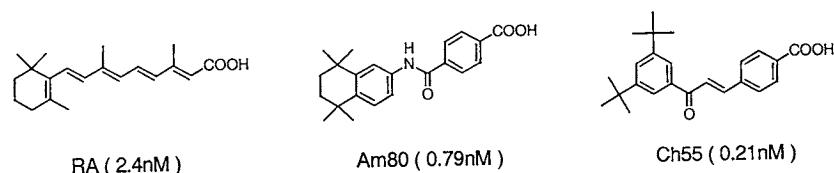


Fig. 2. Structures of RA, Am80 and Ch55.

the use of Am80 and Ch55 labelled with tritium, which do not bind to CRABP efficiently, it has become possible to identify RAR's irrespective of the presence of CRABP. It is only recently that human RAR's have been identified and the features of RAR's have been characterized (*vide infra*) (2).

**II. Retinoic acid receptors (RAR's) as members of the steroid thyroid nuclear receptor superfamily.** Very low concentrations (order of pM) of potent retinoids such as Am80, Ch55 and RA elicit their specific biological effects in various biological assay systems. The signal of such potent retinoid molecules must be amplified without step(s) accompanying increase of entropy (in other words, without scattering or loss of the specificity possessed by the signal molecule). The signal transduction pathway utilizing specific receptor which strongly binds retinoids and binds the specific responsive genes (possibly at the so-called retinoic acid response elements: RARE's) fulfills the criteria for the molecular mechanism of retinoidal actions. We know that such a signal transduction pathway underlies transcriptional regulation induced by small signal molecules such as steroids and thyroid hormones (3). The biological effects of these hormones are now known to be mediated by the nuclear hormone receptors, a superfamily of ligand-dependent transcription factors, which directly link transcriptional responses to extracellular signal molecules.

As specific receptors for retinoids, three subtypes of

RAR's (RAR- $\alpha$ , RAR- $\beta$  and RAR- $\gamma$ ) have been identified so far. RAR's have been cloned as members of the steroid/thyroid nuclear receptor superfamily (3). The established nuclear receptors are: the receptors for glucocorticoid, for mineralcorticoid, for estrogen, for progesterone, for androgen, for 1,25-dihydroxyvitamin D<sub>3</sub>, for thyroxine (two subtypes; TR's), and for retinoids (RAR's). All of these receptors have been established to act as ligand-dependent trans-acting transcription factors. Several other cDNA's encoding proteins belonging to the steroid/thyroid nuclear receptor superfamily but whose corresponding ligand is not known, have also been cloned. Such receptors are called orphan receptors (the author regards the recently cloned RXR which is reported to be activated by RA as an orphan receptor, judging from its ligand-binding selectivity and the concentration of RA necessary for the activation of RXR) (4). Based on the similarity of their amino acid sequence, all of these receptor proteins can be divided into six domains, A-F (ex. RAR's; Fig. 3). Amino acid sequence homology among all of the receptors is high in the C domain (about 50%). This domain also possesses high homology with the part of v-erbA oncogene product (c-erbA is the gene of TR). Each of the six domains possesses separate function(s) and each domain is interchangeable. The general functions of each domain of the steroid/thyroid nuclear receptors are interpreted as described below (3). Roughly speaking, A/B domain encodes transcriptional activating function, C domain encodes DNA-binding function, and E domain encodes

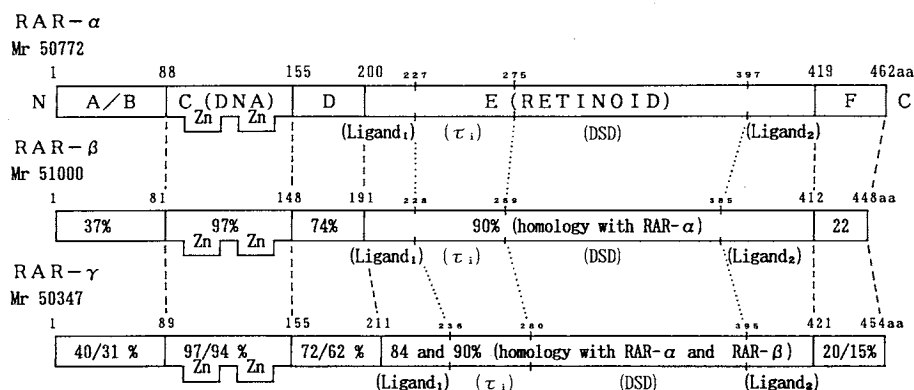


Fig. 3. Gross structures of human RAR- $\alpha$ ,  $\beta$  and  $\gamma$ .

ligand-binding function.

The highly conserved 60 to 70 amino acid C domain which is located within the interior of the protein encodes the base sequence specific DNA-binding function. This domain contains nine conserved cysteines, eight of which form two zinc finger DNA-binding motifs. Discrimination among specific binding sites is mainly determined by three amino acids at the base of the first (positioned nearer to N-terminal) zinc finger. Members of the steroid/thyroid nuclear receptor superfamily can be classified into four groups according to the three amino acids present at these discriminatory positions. In this sense, RAR's belong to a subgroup consisting of TR's and the 1,25-dihydroxyvitamin D<sub>3</sub> receptor. The second zinc finger is considered to stabilize the binding of the receptor to DNA.

Adjacent to the C-terminal of the C domain is the D domain with a short sequence similar to those found in the SV40 T antigen. This domain is considered to be a hinge region, and might be in part responsible for the intranuclear localization of the receptors.

The E domain located adjacent to the C-terminal of the D domain is the ligand-binding region. This domain is also considered to be the site of ligand-dependent dimerization and the site of interaction with other nuclear factors. In addition, this domain possesses a transcriptional inactivating function which is relieved by ligand binding. The intrinsic transcriptional activating function encoded in A/B domain is interpreted to be masked by ligand-free E domain. This transcriptional inactivating function encoded by the E domain is universally effective in some types of non-related transcription factors. For example, the ligand-relieved inactivation can be transferred to c-myc, adenoviral E1A and HIV-1 transactivator Rev simply by fusing these proteins to the E domain of the glucocorticoid and/or estrogen receptors (5). Though the E domain of the estrogen receptor is shown to encode the ligand-relieved inactivating function, this domain has also been shown to possess the ligand-dependent partial transcriptional activating function; the estrogen receptor truncated with A/B domain has been shown to act as a ligand-dependent transcription factor though its efficiency is low (6). The ligand-dependent partial transcriptional activating function of the glucocorticoid receptor's E domain has also been shown.

As such, the E domain seems to be complex structurally and functionally, and may be divided into four putative subdomains which are interchangeable, *i.e.*, Ligand<sub>1</sub>,  $\tau_1$ , dimerization subdomain (DSD), and Ligand<sub>2</sub> (from the N-terminal to the C-terminal of E domain). Ligand<sub>1</sub> and Ligand<sub>2</sub> subdomains are nearly identical among receptors for the same ligand but are different among receptors of different ligand-binding specificity, suggesting the possibility of direct contact of

these subdomains with the corresponding ligand.  $\tau_1$  subdomain is highly conserved among all nuclear receptors and is proposed to encode the transcriptional inactivating function. Inactivation by this subdomain is relieved by ligand binding. DSD consists of highly conserved eight to nine heptad repeats of hydrophobic amino acids, which is similar to a leucine zipper motif. Dimerization of RAR's, and formation of heterodimer with TR or other nuclear factors through this subdomain have been well documented (7, 8).

The function of the C-terminal F domain is not known, but it might be responsible for stabilization of the receptors by forming a C-terminal node.

The N-terminal A/B domain is considered to be a major region of transcriptional activating function. In fact, the deletion of the E domain resulted in a constitutively active ligand-independent receptor in the cases of estrogen, glucocorticoid and 1,25-dihydroxyvitamin D<sub>3</sub> receptors. The transcriptional activating function encoded in the A/B domain is cell-type specific. In the case of the estrogen receptor, deletion of the E domain resulted in a receptor which is constitutively active in one type of cell but is completely inactive in another type of cell (6). The result suggests that there exists cell type-specific co-factor(s) which mediates or inactivates the transcriptional activating function of the A/B domain by interacting with this domain. Several results which suggest the existence of nuclear co-factor(s) which mediates the transcriptional activating function of nuclear receptors have been reported. Such a co-factor is considered to be contained in a cell nuclei by only a limited number of molecules. From the observation of phenomena called squelching (loss of responsibility of the cells toward one steroidal ligand by over-expression of a nuclear receptor for another steroidal ligand), the possibility that such a nuclear co-factor is commonly competed for by all of or at least more than two types of nuclear receptors was proposed. If such a co-factor really exists, it might be a critical factor which uniformly determines the capacity of cells in their response against various ligands for nuclear receptors. There is the possibility that such a co-factor is cell type-specific and determines the hierarchy of response against various ligands for nuclear receptors. Identification of this or these co-factor(s) will no doubt attract greater attention in the future.

*III. Interaction of retinobenzoic acids with RAR's in relation to their biological activities.* Gross structures of human RAR's are represented in Fig. 3 (3, 4). Three subtypes of mouse RAR's have also been cloned. The structure of each subtype of mouse RAR is almost identical to the corresponding human RAR subtype (the interspecies conservation is much higher than the conservation of all three RAR's within one species).

**Table I.** COMPARISON OF ACTIVITIES OF RA, Am80 AND Ch55 IN VARIOUS ASSAY SYSTEMS.

	RA	Am80	Ch55	The Order
Binding to RAR- $\alpha$ ( $K_a$ , $M^{-1}$ )	$3.7 \times 10^{10}$	$2.4 \times 10^{10}$	$\sim 10^{11}$	Ch55 > RA > Am80
Binding to RAR- $\gamma$ ( $K_a$ , $M^{-1}$ )	$\sim 10^{10}$	(< $\sim 10^6$ ) (no binding)	$\sim 10^{11}$	Ch55 > RA
Differentiation-Induction of F9 ( $ED_{50}$ , M)	$3.7 \times 10^{-10}$	$6.3 \times 10^{-9}$	$2.6 \times 10^{-10}$	Ch55 > RA > Am80
Induction of RAR- $\beta$ Expression (relative activity)	1	$\sim 0.3$	$\sim 1.9$	Ch55 > RA > Am80
Inhibition of ODC <sup>a</sup> -Induction (effective dose, M)	$2.0 \times 10^{-9}$	$1.5 \times 10^{-8}$	$1.0 \times 10^{-9}$	Ch55 > RA > Am80
Binding to RAR- $\beta$ ( $K_a$ , $M^{-1}$ )	$2.2 \times 10^{10}$	$2.9 \times 10^{10}$	$\sim 10^{11}$	Ch55 > Am80 > RA
Differentiation-Induction of HL-60 ( $ED_{50}$ , M)	$2.4 \times 10^{-9}$	$7.9 \times 10^{-10}$	$2.1 \times 10^{-10}$	Ch55 > Am80 > RA
Induction of EGFR <sup>b</sup> Expression (effective dose, M)	$\sim 1 \times 10^{-7}$	$\sim 5 \times 10^{-8}$	$\sim 5 \times 10^{-9}$	Ch55 > Am80 > RA
Affinity to CRABP (relative affinity)	1	1/20	no binding	RA > Am80 > Ch55

<sup>a</sup>) ODC, ornithine decarboxylase.

<sup>b</sup>) EGFR, epidermal growth factor receptor.

The amino acid sequence homologies among human RAR- $\alpha$ ,  $\beta$ , and  $\gamma$  range between 94 and 97% for the C domain, and between 84 and 90% for the E domain. The A/B and F domains possess almost no remarkable amino acid sequence homologies among the three RAR's. By the use of oligo-peptides, whose sequences correspond to the A/B or F domains of each RAR as antigens, antibodies specific for each RAR could be obtained.

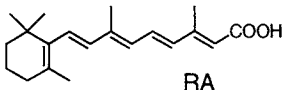
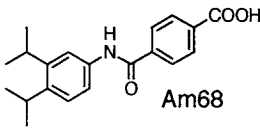
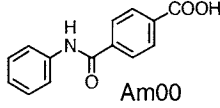
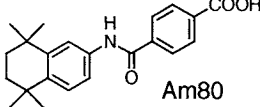
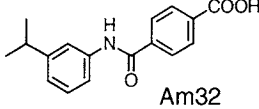
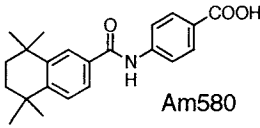
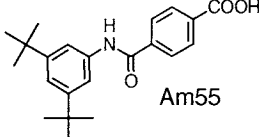
All of RAR's are intranuclear localized as are TR's. RAR's do not possess affinity toward 90 kDa heat shock protein. Human RAR's were first identified in human promyelocytic leukemia cells HL-60 which are induced to differentiate into mature granulocytes by low concentrations of retinoids (2). RAR- $\beta$  can be extracted from cells using a buffer containing low concentrations of salts. On the other hand, extraction of RAR- $\alpha$  requires a buffer containing a high concentration (0.3–0.6 M) of potassium chloride. These RAR's bind efficiently to RA, Am80 and Ch55 with the association constants ( $K_a$ 's) of  $10^9$ – $10^{11}$   $M^{-1}$ . Each RAR can be separated by anion exchange high performance liquid chromatography (HPLC) and can be identified by the use of the antibodies specific for each RAR (2). It is also possible to extract RAR- $\alpha$ ,  $\beta$ , or  $\gamma$  from cells transfected with expression vector bearing each corresponding RAR genes. Investigation using size exclusion gel HPLC suggests that RAR's form dimers ligand-dependently.

Though the amino acid sequence homologies of the ligand binding E domain are quite high (84–90%) among the three subtypes of RAR's, they can be discriminated by the different ligand-binding selectivities. The ligand-binding selectivities of RAR's toward RA, Am80 and Ch55 are shown in Table I (2, 4). Ch55 binds to all of the three RAR's with highest affinity among the three retinoids. For RAR- $\alpha$ , RA binds stronger than Am80, but the order is reversed for RAR- $\beta$ . Amazingly, the potent retinobenzoic acid Am80 seems to possess almost no affinity toward RAR- $\gamma$  (the result is still pre-

liminary). These findings strongly suggest the possibility that retinobenzoic acids which are specific for one or two of the three subtypes of RAR's can be developed. It seems to be also possible to find specific antagonists of retinoids. Actually, some retinoids which possess unique binding selectivity characteristics toward RAR's and some retinoid-antagonists are now being created.

In cell differentiation induction assay using HL-60 cells, Am80 is more potent than RA. This order of biological potency coincides with the order of ligand-binding affinity toward RAR- $\beta$  but not with that toward RAR- $\alpha$  or  $\gamma$  (Table I). Rather good co-relation between  $ED_{50}$  values for HL-60 cell differentiation assay and inhibition constants ( $K_I$ 's) for binding to RAR- $\beta$  are recognized for a series of retinobenzoic acids (Fig. 4). Though we cannot say generally that the potency of biological activities should correspond to the binding affinity of compounds to their receptor (ex. antagonists break the correlation), it may be tentatively assumed that RAR- $\beta$  plays an essential role in the retinoid-induced cell differentiation of HL-60. In addition, the major retinoid-specific binding activity of HL-60 cells is due to RAR- $\beta$  (RAR- $\alpha$  is minor) (2). Of course, the author does not deny the role of RAR- $\alpha$  in the cell differentiation of HL-60 cells induced by retinoids. Results which indicate that RAR- $\alpha$  also plays an important role in the retinoid-induced cell differentiation of HL-60 have been reported.

The presence of both RAR- $\alpha$  and  $\beta$  in HL-60 cells has been well established by the author and co-workers (2). However, several investigators reported that only RAR- $\alpha$  is expressed in HL-60 cells, judging from the analysis of RAR-transcripts. There are various possible explanations for this discrepancy: (a) RAR- $\beta$  mRNA is more unstable than RAR- $\alpha$  mRNA in HL-60 cells. Such exceptional instability would make the detection of RAR- $\beta$  mRNA in HL-60 cells very difficult. A shorter half life time of RAR- $\beta$  mRNA than that of RAR- $\alpha$  mRNA has been reported. (b) The efficiency of translation of RAR-

	ED <sub>50</sub> (nM)	K <sub>i</sub> (RARβ) (nM)		ED <sub>50</sub> (nM)	K <sub>i</sub> (RARβ) (nM)
	2.4	4.5		2.1	24
	inactive	15000		0.79	2.3
	680	1000		0.34	0.24
	36	100			

**Fig. 4.** Activities (ED<sub>50</sub> values) of an amide series of retinobenzoic acids and their inhibition constants (K<sub>i</sub> values) for RAR-β measured by means of the binding competition with Am80.

β mRNA is higher than that of RAR-α mRNA in HL-60 cells. This would result in a higher amount of RAR-β translated from a low level of RAR-β mRNA than that of RAR-α translated from a high level of RAR-α mRNA. (c) The level of RAR-β mRNA expressed in the HL-60 cells used by the author and co-workers is higher than that expressed in the HL-60 cells used by the other investigators. Such an explanation is possible because the author and co-workers use a cloned HL-60 cell subline which possesses high responsibility toward retinoids. Differences in the features among HL-60 sublines maintained in various laboratories are well known. In addition, expression of RAR-β mRNA is established to be induced by retinoids. The result suggests the possibility that our HL-60 cells were induced to express a high level of RAR-β mRNA by the very small amount of RA contained in the fetal calf serum used for maintenance of the cells.

As mentioned above, the order of potency of RA, Am80 and Ch55 in HL-60 cell differentiation induction assay coincides with the order of binding affinity toward RAR-β of these three retinoids. Another assay system which shows a coincidence of the order of potency with the order of binding affinity toward RAR-β is the retinoid-induced expression of the receptor of epidermal growth factor (Table I). On the other hand, the order of potency of RA, Am80 and Ch55 in several other biological assay systems coincides with the order of binding affinity toward RAR-α; *i.e.*, retinoid-induced cell differenti-

ation of mouse embryonic teratocarcinoma cells F9, retinoid-induced expression of RAR-β gene, and inhibition of ornithine decarboxylase activity induced by a tumor promoter (Table I). The author assumes that the difference of the order of potency of RA, Am80 and Ch55 observed in various biological assay systems is due to the difference of the RAR-subtype which plays an essential and/or a direct role in the assay system. In this sense, the discovery of retinoidal activities which are elicited by RA and Ch55 but not by Am80 (lacking affinity toward RAR-γ) will offer information concerning the role played by RAR-γ. One such biological response is skin irritation. Application of retinoids generally induces skin irritation, but Am80 does not induce such a response efficiently. The observation suggests that the RAR which is responsible for the retinoid-induced skin irritation is RAR-γ. RAR-γ is known to be distributed in skin specifically in adult mammals. From the clinical standpoint, retinoids which do not bind or activate RAR-γ, such as Am80, might be superior for the treatment of dermatological diseases.

There has been no biological assay system in which the order of potency of RA, Am80 and Ch55 coincides with the order of binding affinity of these three retinoids toward CRABP, indicating that CRABP is not essential for retinoidal actions.

**IV. Distribution of RAR's and their hypothetical roles.** Three subtypes of human RAR's are encoded

by different genes located on human chromosome 17 for RAR- $\alpha$ , on chromosome 3 for RAR- $\beta$ , and on chromosome 12 for RAR- $\gamma$ . There exist several mRNA isoforms for each RAR due to the different splicing sites (there is no evidence which indicates the presence of all of the corresponding isoforms as protein). The different spatial distributions of mRNA of these three subtypes and of several isoforms have been analyzed. However, it may be more important to analyze the distribution of each RAR protein, not that of their transcripts. Actually, in the case of HL-60 cells, the amount of RAR- $\beta$  mRNA detected is much lower than that of RAR- $\alpha$  mRNA, though the majority of RAR in the cells is RAR- $\beta$  (*vide supra*).

The author and co-workers established the analytical method of RAR's in tissues or cells. Briefly, the RAR's are extracted using a buffer containing 0.6 M potassium chloride, incubated with tritium labelled retinoids such as [ $^3$ H] Am80 and [ $^3$ H] Ch55, and the mixture was analyzed by anion exchange HPLC equipped with radioisotope detector. The analysis of adult mouse tissues offered several kinds of information concerning the characteristic distribution of each RAR, though the results are still preliminary: (a) RAR- $\alpha$  exists universally in all of the tissues analyzed. (b) RAR- $\beta$  exists in rather restricted tissues. For instance, RAR- $\beta$  protein is not found in the brain or small intestines. (c) Distribution of RAR- $\gamma$  is highly restricted. RAR- $\gamma$  protein was found only in the skin tissue, though the identification of the receptor is not conclusive. These features of distribution of RAR's are summarized in Table II. Similar results have been obtained for specificity of distribution of each RAR in regenerative newt tissues and in mouse tissues in the embryogenetic stage.

The temporal distribution of each RAR is also considered to be specific. The expressions of RAR- $\alpha$  and  $\gamma$  are steady but the expression of RAR- $\beta$  can be induced by retinoids. In addition, RAR- $\beta$  itself and its mRNA are less stable than the other two RAR's and their transcripts, respectively. The author proposes the following hypothesis concerning the specificity of the roles played by peculiar RAR: (a) The specificity of the roles played

by RAR- $\gamma$  is due to the highly specific and restricted distribution of the receptor. (b) The specificity of the roles played by RAR- $\beta$  is due to its temporally specific expression. The retinoid-induced expression of RAR- $\beta$  gene suggests that RAR- $\beta$  is expressed only when there is available retinoid molecules. The universal and steady expression of RAR- $\alpha$  might suggest that RAR- $\alpha$  plays a role in detecting the presence of available retinoid molecules, and in transactivating the expression of RAR- $\beta$  gene in the cells which need the receptor (*vide infra*). Such a mechanism might be present because of the disadvantages caused by the constitutive expression of RAR- $\beta$  (over-expression of RAR- $\beta$  caused by insertion of a hepatitis B virus gene is known, though the relation between abnormal cell proliferation and the over-expression of the gene is unclear). (c) As such, RAR- $\alpha$  might act as a sensory molecule for retinoids. Therefore, RAR- $\alpha$  might be essential for the initial event of retinoidal actions at least in some of the various biological responses against retinoids. Such a consideration makes it possible to explain why the abnormality or over-expression of RAR- $\alpha$  results in the loss of responsibility toward retinoids of the cells. Abnormality of RAR- $\alpha$  is reported for HL-60 cell subline which is resistant to retinoids. Expression of abnormal fused protein due to the translocation of the specific chromosome fragment into the RAR- $\alpha$  gene locus in acute promyelocytic leukemia cells has also been reported.

The morphogenic action of retinoids, that is, the determination of polarity of pattern formation by retinoids, might be better interpreted by the specific distribution of RAR subtypes or RAR isoforms, rather than by the morphogen gradient theory (9). The morphogen gradient theory states that cells in a developing embryo measure the concentration of the morphogen so that the cells can determine their position relative to a specific landmark. Accurate positional information is without doubt important for development. At a glance the reported detection of the positional difference of RA concentrations (9) seems to support the theory. According to the morphogen gradient theory, RA acts as a signal molecule to provide the cells with the positional infor-

Table II. COMPARISON OF HUMAN RAR- $\alpha$ ,  $\beta$  AND  $\gamma$ .

	RAR- $\alpha$	RAR- $\beta$	RAR- $\gamma$
Chromosome	17	3	12
Number of Isoforms <sup>a)</sup>	2-isoforms	3-isoforms	2-isoforms
Amino Acids of the Major Isoform	462	448	454
Distribution	universal	rather restricted	highly restricted
Expression	steady	induced by retinoids	steady
Stability of the Protein	stable	unstable	stable
Stability of the Transcripts	stable	unstable	stable

<sup>a)</sup> Isoforms possessing different amino acid sequences.

mation. However, the author does not understand how the information (the gradient, that is the continuous difference of RA concentration) can be altered to the discontinuous signals which switch on or off the specific gene transcription. The pattern of gene expression of each cell under development should not be vague or continuous. Therefore, it is better to interpret the responsibility of cells to RA as intrinsically pre-determined by the specific distribution of each RAR. In fact, in spite of the potent activities of Am80 and Ch55 as morphogens like RA in pattern formation assay using an embryonic chicken limb bud (10), no gradient of the concentration of Am80 or Ch55 was detected in the embryonic chicken limb bud. The gradient of RA detected in the embryonic chicken limb bud might be due to the restricted distribution of CRABP and/or RAR's. Recently, experimental results which suggest that retinoids act not as morphogens but as inducers of ZPA (zone of polarizing activity) have been reported.

*V. Genes whose expression are regulated by retinoids.* Retinoids elicit diverse biological effects by regulating the expression of various genes. The regulation of the gene expression is now established to be mediated by the RAR's.

The expression of more than fifty genes are reported to be regulated in the cells treated with retinoids (4). Such genes include homeobox-containing genes, oncogenes, nuclear receptor genes (RAR- $\beta$  and progesterone receptor genes), the genes of transcription factor AP-2 and of the related nuclear factors, the genes of growth factors and of growth factor receptors, the genes of some enzymes and cytoskeleton-related proteins, CRABP genes, and so on. It is likely that the majority of the genes listed above are not transcriptionally regulated by retinoid-bound RAR's (holo-RAR's) directly. Actually, the products of almost half of the genes listed above are considered to be transcription factors or related factors, which will further regulate the expression of other genes. Judging from the low concentration of retinoids effective to induce the specific cellular responses and from the low amounts of RAR's in cells, at least some of the primary targets are probably transcription factors or related factors which amplify the initial event(s) caused by holo-RAR(s). The transcription of the primary target gene(s) is assumed to be regulated by the direct binding of the holo-RAR(s).

In spite of the accumulation of reports treating genes whose expression are indicated to be regulated by retinoids, the expression of only ten genes have so far been established to be transcriptionally regulated by holo-RAR's directly. For these genes, the sequences which exist in the 5'-flanking region of the genes and which confers the responsibility against retinoids on the genes, have been identified. Such sequence(s) is called the RA

response element (RARE). RARE's seem to be the binding sites of holo-RAR's, judging from gel retardation experiments and the recognition of the retarded bands containing RARE-sequences by the RAR-specific antibodies. However, there is no evidence which indicates that RAR directly binds to RARE-oligodeoxyribonucleotide. To show the direct binding of RAR to RARE will require experiments using purified RAR's.

The characterized RARE's are in the promoter region of RAR- $\beta$  gene, of lamini B1 gene, of pituitary growth hormone gene, of epidermal growth factor receptor gene (c-erbB1) and the related gene c-erbB2/neu, of thyrosine kinase gene, of myosin heavy chain gene, of complement factor H gene, of osteocalcin gene, and of vitellogenin gene (2, 3). Among RARE's of these retinoid-responsive genes, that of the RAR- $\beta$  gene seems to be the only genuine RARE for RAR's. RARE's of the other genes are common (or overlapped) for the response element of TR (TRE) (*i.e.*, RAR's bind to TRE), of 1,25-dihydroxyvitamin D<sub>3</sub> receptor, of transcription factor AP-1, or of estrogen receptor (11). As such, RAR's seem to recognize various response elements, some of which are common for different classes of nuclear receptors and of transcription factors. (Of course, not all of the TRE's and other response elements for other nuclear receptors and for factors are necessarily recognized by RAR's.) Generally speaking, several different classes of transcription factors and their regulators might modulate expression of a single gene by binding to a common or overlapped sequence. This phenomenon might provide an explanation for the interplay of growth and differentiation factors, which is referred to as cross-coupling (or cross-talk). Such a molecular mechanism of cross-coupling is different from that of the inhibition of the function of AP-1 with glucocorticoid receptor by the direct protein-protein binding of AP-1 to the receptor.

Almost all of the sequences of RARE's (and TRE's and other response elements which are common for retinoids) contain a palindromic or a half palindromic motif, suggesting the binding of RAR's as a dimer. However, the sequence of a RARE in RAR- $\beta$  gene contains an unusual direct repeat of GTTCAC which is somewhat related to the half palindromic motif GGTCAC of TRE and estrogen response element. It might be possible that RAR's bind to the RARE in RAR- $\beta$  gene as a monomeric form.

*VI. Interactions of RAR's with other proteins through its C-terminal E domain.* The interplay between retinoids and thyroid hormones has been well documented. The results indicate that these two classes of substances involved in morphogenesis can control overlapping gene networks, and thus exert a concerted regulation on developmental events. The interplay be-



tween RA and thyroxine seems complex. For example, the transcription from a TRE of some thyroxine responsive genes is enhanced by holo-RAR's, and the effect of holo-RAR's is repressed by ligand-free TR (apo-TR). On the other hand, holo-TR enhances the transcription from the same TRE even in the presence of apo-RAR's. Results which indicate that binding of RAR's to some RARE's or to TRE's is increased by addition of TR have been obtained. In addition, the heterodimer formation consisting of RAR and TR *in vitro* is well established (7). Such heterodimers have been established to be formed through the heptad repeat motif which exists in the E domain of the receptor (Fig. 3) (3, 7). The heterodimers were reported to bind to TRE's with enhanced affinity, and to produce either transcriptional stimulation (for palindromic TRE's) or transcriptional inhibition (for a myosin heavy chain TRE) according to the precise sequence of the TRE's. It is possible that such heterodimers are also formed *in vivo*. It is also possible that nuclear receptors may also interact with other members of the steroid/thyroid nuclear receptor superfamily or with modulators, resulting in novel patterns of gene expression.

Actually, the existence of multiple cell type-specific nuclear DNA-binding factors (referred to as RAR-coregulators) which bind to RAR- $\alpha$  at its heptad repeat motif has been reported (7). The RAR-coregulators have been proposed to differentially regulate the binding affinity of the RAR- $\alpha$  for a variety of RARE's. According to the report, HeLa cells contain the RAR-coregulators of 50 kDa and of 65 kDa, HL-60 cells contain those of 45 kDa and of 55 kDa, and pituitary tumor GC cells contain TR and the RAR-coregulator of 58 kDa, as major proteins which interact with RAR- $\alpha$ . Interaction (binding) of these distinct RAR-coregulators existing in different cell types are proposed to confer a distinct hierarchy of binding site affinities within the different cell types on RAR- $\alpha$  (*i.e.*, the hierarchy of binding site affinities of RAR- $\alpha$ ) which is as follows; RAR- $\beta$  RARE (RARE- $\beta$ ) > a palindromic TRE (TRE-pal) > a myosin heavy chain TRE (TRE-mhc) for HeLa cells; TRE-pal > a laminin B1 RARE > TRE-mhc for HL-60 cells; and TRE-mhc > TRE-pal >> RARE- $\beta$  for GC cells. Such RAR-coregulators are considered to regulate target sequence recognition by RAR- $\alpha$  differentially according to the difference of cell type. In other words, the difference in the sets of target genes, whose transcription is distinctly regulated by retinoids according to cell type, is pre-determined by the presence of such multiple cell type-specific RAR-coregulators. The presence of the RARE which functions in some cell types but not in others can also be explained by the existence of such RAR-coregulators.

**VII. Diversity of retinoidal activities.** Taking altogether the results and discussion the author described above, the molecular mechanism of retinoidal actions can be represented as shown in Fig. 5. There are three subtypes of RAR's ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), all of which are nuclear-localized ligand-specific transcription factors. RAR's are the members of the steroid/thyroid nuclear receptor superfamily, and are divided into six domains (domains A~F). The transcriptional activation function is encoded in the A/B domain, whose function is masked by the ligand-free E domain. Some cell type-specific co-factors which regulate the function of the A/B domain by binding to this domain might exist. The sequence-specific binding activity through the zinc finger motifs at the C domain is interpreted to also be masked by the ligand-free E domain. These masking effects are relieved by the binding of a retinoid molecule which arrives at the nuclei by diffusion. The binding site of retinoids exists in the E domain. The binding of the ligand promotes the dimer formation of the RAR through the heptad repeat motif existing in the E domain. The dimer which is ligand-dependently formed can be either a homodimer or a variety of heterodimers; *i.e.*, RAR's form heterodimers with TR's or various cell type-specific RAR-coregulators which exist in the nuclei. According to the constituents of the heterodimer, the complex recognizes distinct specific base sequences and controls the gene expression.

Retinoids elicit a wide variety of specific biological effects. The diversity of the retinoidal actions may be due to the diversity of RAR's, of RARE's and RAR-coregulators, of the nuclear co-factors, and of retinoid responsive genes. Possible interpretations are as follows. (a) The qualitative and distributional diversity of RAR's is the source of the distinct responses against retinoids of the different cells. The distinct RAR's possess different ligand binding selectivities, and may possess different hierarchies of binding affinities for various RARE's. (b) The diversity of the base sequences of RARE's is another source of difference, together with the diversity of the cell type-specific RAR-coregulators which determine the hierarchy of binding affinities of RAR's to RARE's. Though the roughly measured molecular mass and the DNA-binding activity of some of the putative RAR-coregulators are reported, the detailed nature of these proteins are unknown. The identification and characterization of such RAR-coregulators are an important working hypothesis. (c) The diversity of the nuclear co-factor(s) which may bind to the A/B domain and may regulate the function of this domain may result in the diverse responses of different cells against retinoids. The diversity of these co-factor(s) might be either qualitative or distributional. The presence itself of such co-factor(s) is still hypothetical. However, there are several lines of experimental evidence

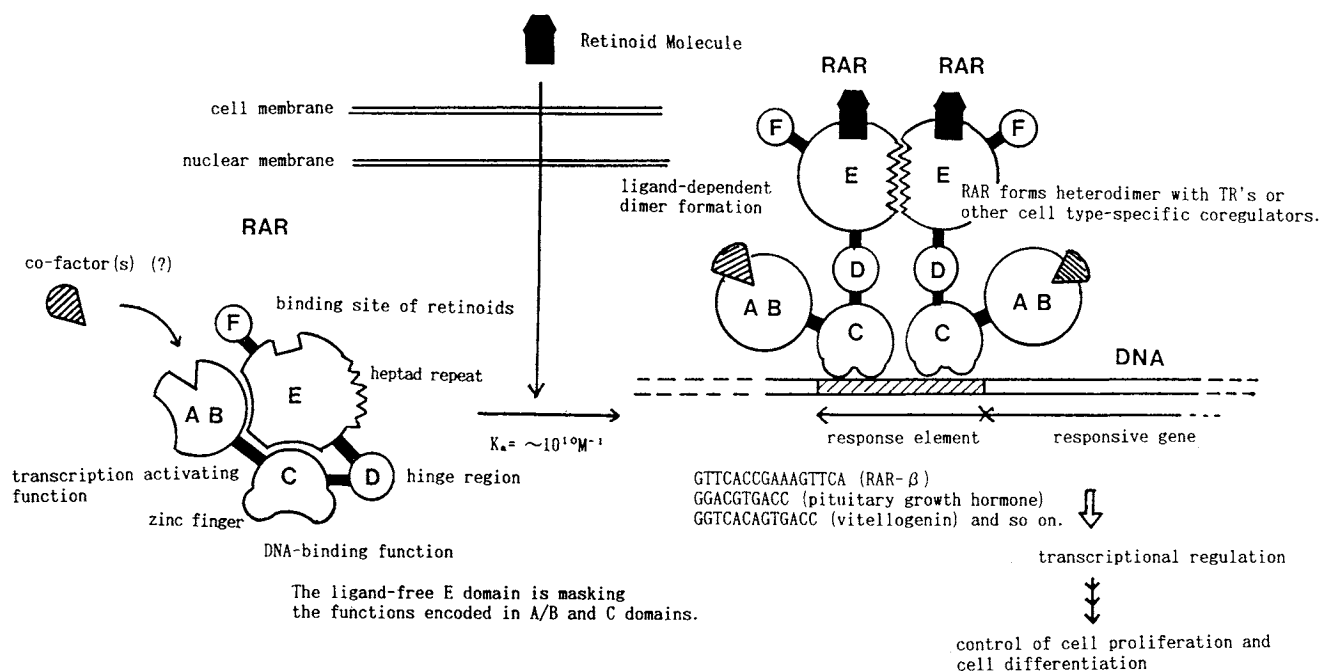


Fig. 5. The molecular mechanism of transcriptional regulation initiated by retinoids.

which suggest that this co-factor may be common for plural types of nuclear receptors and for other classes of transcription factors, as mentioned before. It would also be of great interest to identify and characterize such co-factors. (d) The diversity of the products of the various retinoid-responsive genes is without doubt what makes the observed retinoidal action pathways extremely complex. Expression of the many genes of transcription factors and of the related factors are regulated by retinoids. The products of these genes further regulate the expression of their specific responsive genes. The cascade and the hierarchy of such transcriptional control are considered to be cell type-specifically predetermined.

Though many retinoid-responsive genes have been identified so far, the author suspects that there exists a primary target gene(s) other than the known retinoid-responsive genes. Judging from the mode of retinoidal actions and the quite low retinoid-concentration which is biologically effective, the primary target gene(s) of retinoids would encode a transcription factor (or the related factor) which is critical for cell proliferation and cell differentiation. Because the potent retinoids are effective at the concentration corresponding to a few molecules per cell, the initial event caused by such retinoids must be amplified efficiently probably through the transcriptional control of the specific transcription factor(s). Identification of such primary targets is essential for the elucidation of the molecular mechanism of retinoidal actions.

**VIII. Concluding remarks.** Retinoids are now recognized as one of the most biologically important classes of compounds. The author reviewed in this article our recent progress in developing novel potent retinoids, retinobenzoic acids, and their application in the investigation of RAR's. In addition, the investigations concerning the molecular mechanism of retinoidal actions were reviewed. The molecular mechanism of the initial event caused by retinoids explained in this article (summarized in Fig. 5) is the most simple and important signal transduction pathway both theoretically and biologically.

The observed responses of cells against retinoids seem to be extremely complex at a glance, and to be hard to interpret at the molecular level. However, our knowledge is still quite restricted, and the molecular mechanism of the whole retinoidal action will become more simply explicable with the accumulation of advanced investigations and by their organized interpretation.

For the more detailed research on the retinoidal actions, it will be fruitful to develop novel retinoids specific for the distinct RAR's and their antagonists (some such compounds have already been prepared). Such compounds would be useful not only as the tools of biochemical and biological research but also as the lead compounds for the development of superior retinoidal medicines. Detailed analysis of the interaction of the various retinoids with RAR's will make the molecular design of useful specific compounds more accessible.

For this purpose, mass-preparations of RAR's and/or their recombinant products aiming at crystallization by the use of *E. coli* and/or other expression systems are also under way. Methods for analysis and short step purification of RAR's from various tissues or cells are also being established.

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