CMLS, Cell. Mol. Life Sci. 61 (2004) 2309–2321
1420-682X/04/182309-13
DOI 10.1007/s00018-004-4065-4
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CMLS Cellular and Molecular Life Sciences

Review

Receptors for steroid hormones: membrane-associated and nuclear forms

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Received 11 February 2004; received after revision 7 April 2004; accepted 26 April 2004

Abstract. Steroid hormones are now recognized to act through both nuclear and membrane-associated receptors. In this review we focus on receptors for estrogen and the vitamin D metabolite 1,25-dihydroxyvitamin D_3. While the nuclear receptors are part of a 'superfamily' with common structural elements, membrane receptors are more diverse, ranging from variants of the nuclear forms to unrelated proteins. We conclude that both rapid (membrane-initiated) actions, as well as regulation of gene transcription, are necessary to explain the complex actions of steroid hormones on target cells.

Key words. Estrogen; $1,25(OH)_2D_3$; membrane receptors for steroid hormones; nuclear receptors; rapid responses; transcriptional regulation.

Introduction

Prior to the late 1950s, most information on the actions of steroid hormones derived from biological observations of changes in animal model systems after hormone administration. In particular, investigators observed remarkable changes in uterine size, due to increased fluid and ion permeability, and blood flow following estrogen administration in estrogen-withdrawn rats (reviewed in [1]). Based on extensions of these observations, Szego and Roberts [2] hypothesized in 1953 that this increase in permeability was the primary mechanism of action of hormones in general, thus beginning the search for membrane-initiated steroid hormone effects. In the vitamin D field, this theme appeared in the work of Toffolon et al. [3], who reported that a brief $1,25(OH)_2D_3$ treatment in vivo stimulated calcium transport in the rat intestine in vitro. However, during most of the 1960s, observations from the budding area of molecular biology demonstrated hormonal effects on RNA, DNA and protein synthesis, and thus most investigators focused on nuclear actions of the steroid hormones until very recently. This review will provide perspectives on both the membrane-initiated and nuclear transcription effects of steroid hormones. Since it is not possible to summarize observations for all these systems, this review will focus on estrogen and vitamin D receptors (abbreviated ER and VDR, respectively).

It is now widely recognized that steroid hormones mediate actions independent of de novo RNA and protein synthesis. The search for membrane-associated receptors began with the identification of phenomena that were either too rapid to be a result of gene regulation or that used classical membrane-initiated signaling systems. Consequently, a substantial body of literature exists documenting these effects, while the molecular identities of the receptors are more nebulous. For some systems, the receptor that mediates the signaling phenomenon is either equivalent to, or a variant of, the classical nuclear receptor. In other cases, steroid hormones appear to bind effector proteins unrelated to their classical receptors. As

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postulated in an earlier review [4], it is possible that multiple proteins are involved in eliciting the pleiotropic responses. A further order of complexity might arise by heterodimerization of some of these proteins. In this section we will review studies on molecular entities that have been proposed to mediate the actions of ligand. Thus, for membrane-associated estrogen receptors we will first cite evidence for systems in which the classical ER is involved, either directly in response to estrogen or non-estrogen signals. This will be followed by identifications based on pharmacology, proteins that are identified as non-classical ERs, and, finally, receptors that recognize liganded serum transport proteins. A similar format is followed for $1,25(OH)_2D_3$ with a brief additional consideration of another vitamin D metabolite, $24,25(OH)_2D_3$, known to have rapid effects.

Membrane receptors for estrogen and vitamin D metabolites

Estrogen

Reports of the membrane-initiated signaling of the steroid estradiol through cyclic AMP (cAMP) date back to 1967 [5]. As with many steroid hormones in recent reports, signal transduction pathways have been described for various cell types, although a correlational, physiological endpoint has often not been described.

Classical ER at the membrane

It was not until 1977 [6] that specific binding of estradiol-17β to plasma membranes through affinity interactions was found. Confirmation of binding through use of radioisotopically labeled steroid was reported in 1980 [7]. As a result of these studies it was proposed that a protein with high homology to the nuclear receptor was localized at the membrane. Proof was obtained in pituitary tumor cells using anti-ER antibodies, with varying epitope specificities, in immunocytochemical studies [8, 9]. Activation of the surface receptor resulted in estrogen-induced release of prolactin (a physiologically relevant endpoint). Using an anti-idiotypic antibody, a similar conclusion regarding the plasmalemmal ER identity was made for osteoblasts, although only calcium channel signaling was monitored [10].

In recent years, vasodilation mediated by estradiol-stimulated endothelial nitric oxide synthase (eNOS) has provided a physiologically significant system for study. Most of the observations have concerned ERα [11–14], although ERβ has also been found in caveolae, and is able to activate eNOS [15]. Overexpression of ERα and ERβ in Chinese hamster ovary cells indicated that a single transcript gave rise to both nuclear and membrane-associated forms [14]. Site-directed mutagenesis has indicated that serine at amino acid 522 is important for membrane-initiated signaling, but not for nuclear-initiated signaling [13].

A number of the signaling pathways initiated by estradiol interaction with its membrane-localized receptors are classically associated with a requirement for G proteins. However, the ERs lack the traditional serpentine, membrane-spanning motif associated with G-protein-coupled receptors. Intriguingly, cotransfection of COS cells with plasmids containing eNOS, ERα and $G_{\alpha i}$ (but not other G protein variants) resulted in a threefold stimulation of activity [16]. This presents the intriguing question of whether or not an additional 'scaffolding' protein may be necessary to facilitate interaction in the signaling pathway between the soluble ER and membrane-resident G proteins. Additional studies have demonstrated that liganded ER induced co-localization of activated MAP kinase [17], while others have found a shortened form of the ER preferentially localizes in the membrane to activate signal transduction [18].

The human mammary cancer cell line MCF-7 exhibits estradiol-dependent growth. Activation of the mitogen-activated kinases, erk-1/2, occurs in response to estradiol, with c-src activation serving as a proximal event [19]. On the basis of pharmacological inhibitors such as ICI 182780, the authors have concluded that the classical ER is responsible for signal transduction [19]. In contrast, using tamoxifen, others have concluded that neither ERα nor ERβ is involved in estradiol-mediated stimulation of protein kinase C (PKC) activity [20].

Modulation of ER activation in response to non-estrogen signals

In addition to ligand binding to ER in order to initiate signaling, unrelated agonists can activate the ER through phosphorylation [21]. Adrenergic stimulation has also been found to inhibit estradiol:ER-directed gene activation in vascular smooth muscle cells, while β-AR antagonists reverse it [22].

Identifications made on a pharmacological basis

The pharmacology of ER agonists and antagonists has also been used to support the assertion that some membrane-associated receptors responsive to estradiol are not the classical receptor known to be a transcription factor. In osteoblasts, estradiol rapidly activates signal transduction pathways such as calcium influx and the production of the phospholipid mediators inositol triphosphate and diacylglycerol [23]. Tamoxifen did not block estradiol-mediated stimulation, and the authors proposed a different class of receptors [23]. Similarly, estradiol-induced calcium oscillations in pancreatic α-cells and β-cells were not blocked by ICI 182780 [24]. A note of caution

in these interpretations should be added: transfected ERs have been shown to have a different pharmacology than endogenous ERs [25], while others [26] have proposed alternative binding sites on classical receptors to explain differences in ligand preferences. However, in the previously mentioned study [24], additional pharmacological studies provided evidence that the binding site was shared by epinephrine, norepinephrine and dopamine [24], suggesting more strongly that a nonclassical estrogen receptor was found.

Neuroendocrinology has yielded some striking examples of membrane-initiated steroid signaling in the modulation of ion channels. Estradiol has been shown to decrease the function of serotonin receptors through pharmacologically distinct ER in hippocampus vs frontal cortex [27].

Non-classical ERs

In comparison to systems where the membrane receptor for estrogen has been shown to be ERα or ERβ, examples have come to light in which the steroid binds a distinct and unrelated protein. By monitoring the Maxi-K channel in oocytes, it was found that estradiol binds directly to the β subunit [28]. Others have found that the ER interacts with the regulatory subunit of phosphatidylinositol-3-OH (PI-3) kinase [29].

An endoplasmic reticulum-associated protein, Erp60, is a chaperone with high homology or identity to protein disulfide isomerase (PDI), and was originally termed PLC-α [30]. This protein, or a related protein, has been proposed to be a mediator of estrogen action [31]. Indeed, PDI has been reported to bind both estradiol and thyroid hormone [32]. This may represent a recurring theme for some aspects of steroid hormone action.

Cytoskeletal elements also have the potential to represent a unifying aspect of non-nuclear actions of steroid hormones. Binding of estradiol to cytoskeletal elements [33] and microtubules [34, 35] has been observed. In the latter report [35], nanomolar concentrations were found to inhibit microtubule polymerization by >70%. Using immobilized estradiol in an affinity column, Ramirez et al. [34] specifically retained glyceraldehyde-3-phosphate from the eluate. It was subsequently demonstrated in the same work that nanomolar concentrations of 17-β-estradiol stimulated catalysis by the enzyme, whereas progesterone inhibited catalysis. Others have reported [36] binding of estradiol to hsp-27 in New World primates, species which are resistant to gonadal steroid compared to Old World primates.

Membrane receptors that recognize serum transport proteins

Sex hormone-binding globulin (SHBG) is a serum transport glycoprotein that binds certain steroids, which in turn bind to a specific receptor on cell membranes [37]. In both prostate cells [38] and MCF-7 breast cancer cells, estradiol-liganded SHBG activated the cyclic AMP signal transduction pathway. In the prostate cell system, signaling has been linked to secretion of prostate-specific antigen [38].

These findings illustrate the growing complexity of the problem in elucidating the mechanisms of steroid hormone action. Aside from classical ERE signaling from the 5′-promotor region of a gene, estradiol can activate signaling for rapid responses (e.g. secretion) through the classical receptor located at the plasma membrane, activate non-classical membrane receptors that bind steroid directly or through a serum transport protein, and can directly modulate intracellular proteins (enzymes and cytoskeletal elements).

Vitamin D metabolites

The most studied vitamin D metabolite, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], is synthesized when the organism is deficient in vitamin D, calcium and phosphate. Numerous studies have detailed the direct effects of this metabolite in terms of a variety of rapid responses [39–42], while less has been reported regarding the receptor activity involved.

Classical VDR at the plasma membrane

Initially, an absence of classical VDR was reported in purified chick basal lateral membranes, as judged by Western analyses [43]. More recently, a communication has claimed that a subfraction of plasma membrane – caveolae – do contain VDR [44]. However, several points require clarification to support this contention: a demonstration of enrichment for marker proteins of caveolae, proof that the presence of the VDR is not due to a homogenization artifact and demonstration that the VDR is functionally linked to a physiologically important response or even a signal transduction event. The last criterion was attempted by using mice in which the VDR was mutated to lack the DNA binding domain, yielding a phenotype equivalent to the VDR knockout mouse [45]. While the authors concluded that the VDR was responsible for both membrane-initiated and nuclear-initiated events, they failed to consider secondary effects. In particular, there may be a need for either VDR- or calcium-directed nuclear expression to produce a cell that is biochemically competent to respond rapidly to $1,25(OH)_2D_3$. This question might be more satisfactorily addressed with RNA interference experiments in which knockdown is achieved either transiently or in a stably transfected cell line. Interestingly, others have found that in osteoblasts derived from VDR knockout mice, $1,25(OH)_2D_3$ is capable of eliciting calcium fluxes/channel activation, leading the authors to conclude that the classical receptor is not necessary for membrane-initiated events [46].

An antisense approach was used to connect the VDR with myeloid cell differentiation [47]. Transfected cells failed to activate PI-3 kinase, which in turn was found to be necessary for the expression of the surface marker CD14 [47]. Moreover, in the same work the authors reported immunoprecipitation of complexes containing both PI-3 kinase and VDR [47].

Whether or not a cell relies on classical VDR for membrane-initiated signaling may be dependent on tissue specificity. While fibroblasts have been reported to be devoid of plasma membrane VDR [48], others have found that $1,25(OH)_2D_3$ induces translocation of the VDR to the plasma membrane in skeletal muscle [49]. Since no convincing evidence exists supporting the notion that steroid hormones freely diffuse through the membrane, this leaves us with the question of which non-VDR protein in the plasmalemma first bound the steroid to initiate the signal transduction events that led to the VDR translocation. A similar question arises in regard to a report in which investigators found that myotube Src activation in response to $1,25(OH)_2D_3$ resulted in phosphorylation of the VDR [50]. It is not known whether binding of steroid to membrane-associated VDR or another membrane-associated protein resulted in Src activation.

Identification made on pharmacological basis

$1,25(OH)_2D_3$ is a seco-steroid in which the B ring is broken, allowing conformational mobility. In addition, a side chain presents sites for modification to produce analogs. A number of these analogs have been used to distinguish between nuclear-initiated events and membrane-initiated steroid signaling. In osteoblast-like osteosarcoma cells, analogs that were highly effective in activating calcium channels were not effective in binding to the nuclear receptor, while those compounds exhibiting a high affinity for the VDR failed to activate calcium channels [51]. A similar situation was found in priming of NB4 leukemia cells for monocytic differentiation by membrane-initiated steroid signaling [52]. The actual proteins were not identified in these studies.

Non-classical VDRs

A non-VDR protein was proposed to mediate the effects of $1,25(OH)_2D_3$ in an osteosarcoma cell line lacking the nuclear receptor (ROS 24/1; [53]). These workers subsequently used an affinity analog to identify the binding protein. Although many bands were labeled with $[^{14}C]1,25(OH)_2D_3$ bromoacetate, the authors identified annexin II as the primary membrane receptor candidate [54]. Studies on binding kinetics to annexin II indicated a Kd in the nanamolar range, suggesting physiological relevance [55]. Since annexin is a fusogenic protein for vesicular exocytosis, and a primary response to steroid-initiated events is vesicular exocytosis [56], it is conceivable that the protein plays a role at the plasmalemma.

One of the systems with a readily identifiable physiological endpoint for rapid steroid action is the intestinal epithelial cell, where vesicular transport and exocytosis of calcium and phosphate occurs [57, 58]. Using specific binding of $[^3H]1,25(OH)_2D_3$ as a marker activity, basal lateral (blood-surface) membranes were found to be enriched and used as a starting point for purification of a candidate protein of 64 kd [59]. The N-terminal microsequence of the protein (amino acids 1–20) was used to generate a highly specific polyclonal antibody (Ab 099) by the multiple antigenic peptide (MAP) technique [43]. In immunocytochemical studies, it was found that the putative membrane receptor, now termed $1,25D_3$-MARRS protein (for membrane associated-rapid response steroid binding), apparently redistributes from the basal lateral membrane to the nucleus when exposed to hormone [43]. Moreover, the antibody (Ab 099) inhibited steroid-mediated activation of PKC activity in both intact epithelial cells [43] and membranes from rat chondrocytes [60]. A basal lateral membrane protein of appropriate molecular weight was found to be labeled by the affinity analog $[^{14}C]1,25(OH)_2D_3$ bromoacetate [43].

Support for the physiological importance of membrane-initiated steroid signaling came from the observations that high affinity, cooperative binding to $1,25D_3$-MARRS protein was highest in young, growing animals and correlated with robust stimulation of signal transduction pathways and ion transport across the intestine [61, 62]. Table 1 compares age-dependent changes in receptor kinetics for the $1,25D_3$-MARRS protein and the classical VDR. Hormone-stimulated calcium transport declined significantly between 7 weeks of age and 1 year, as did PKC stimulation, affinity of the 1,25D3-MARRS protein and 1,25D3-MARRS protein levels [61, 62]. In comparison, VDR-binding parameters did not change significantly with age over the same period (table 1).

PKC activation in enterocytes was found to be closely associated with $1,25(OH)_2D_3$-stimulated phosphate uptake [63] and transport [64]. Phosphate uptake in isolated intestinal epithelial cells was subsequently used to establish the functional connection between the $1,25D_3$-MARRS protein, now known to be identical to Erp57, and a physiological response to $1,25(OH)_2D_3$ [I. Nemere et al., unpublished]. Using intestinal epithelial cells transfected with ribozyme (catalytically active nucleic acid) directed against the $1,25D_3$-MARRS messenger RNA (mRNA), hormone-stimulated phosphate uptake, PKC activation and specific binding to membranes were decreased. Protein levels of $1,25D_3$-MARRS were decreased, as judged by Western analyses [I. Nemere et al., unpublished]. There is, therefore, substantial support for the role of $1,25D_3$-MARRS protein in mediating membrane-initiated steroid signaling. However, our report of positively

Table 1. Comparison of binding parameters for $1,25D_3$-MARRS and VDR.

Age	Ca % con	$1,25D_3$-MARRS properties			VDR properties		
		mRNA MARRS/ GAPDH	Protein [1,2] rel dens	K_d [2] (nM)	PKC [1,2] % con	VDR [2] K_d (nM)	Protein [2] B_{max} (fmol/mg)
Males							
7 wk	240 [2]	1.28 ± 0.07	1.00 ± 0.02	0.59 ± 0.1	170	0.35 ± 0.1	31 ± 4
14 wk	190		1.12 ± 0.05	1.03 ± 0.1	120	0.38 ± 0.2	36 ± 5
28 wk	180	0.91	0.86 ± 0.06	0.99 ± 0.1	120	0.29 ± 0.2	31 ± 5
58 wk	150	0.95 ± 0.07	0.64 ± 0.02	2.65 ± 0.8	34	0.30 ± 0.1	23 ± 3
Females							
7 wk	308 [3]	1.28 ± 0.07	1.00 ± 0.07	0.8 ± 0.2 [3]	150 [3]	0.08 ± 0.02 [3]	19 ± 1 [3]
14 wk	184	1.06 ± 0.06	1.05 ± 0.07	1.3 ± 0.2	120	0.20 ± 0.06	30 ± 3
28 wk	170	1.25 ± 0.10	0.97 ± 0.02	1.6 ± 0.2	105	0.06 ± 0.04	26 ± 3
58 wk	153	1.12 ± 0.16	0.91 ± 0.06	1.5 ± 0.2	84	0.11 ± 0.04	29 ± 3

[1] [63].
[2] [61].
[3] [62].

cooperative binding of $1,25(OH)_2D_3$ for this protein [61, 62] could be indicative of heterodimerization and therefore the intimate involvement of either VDR or another non-VDR protein.

Membrane receptors for other vitamin D metabolites

The rapid actions of two additional vitamin D metabolites should be mentioned briefly. The precursor metabolite, 25-hydroxyvitamin D_3 [$25(OH)D_3$] has been found to have a direct effect on isolated intestinal cells [64, 65] and presumably acts through one of the identified binding proteins ([64], and references therein).
Another metabolite, 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$], is made under conditions of $1,25(OH)_2D_3$, calcium and phosphate sufficiency. Not surprisingly, $24,25(OH)_2D_3$ has been found to counteract the rapid effects of $1,25(OH)_2D_3$ on phosphate [58] and calcium transport [66]. Specific binding for this metabolite has been observed both in basal lateral membranes, but to a larger extent in a vesicular fraction [67]. Studies are currently underway to identify and characterize the binding protein.

Nuclear transcription factor receptors

The most well studied pathway for the mechanism of action of receptors for steroid hormones and most other lipophilic agonists is through their functions as ligand-activated nuclear transcription factors [1, 68–73]. Studies of these mechanisms span nearly 50 years, with many investigators contributing to each incremental advance in knowledge along the way. Moreover, while these mechanisms were first recognized for traditional steroid hormones, recent studies have established their validity for many other lipophilic ligands (table 2). Finally, there are many proteins in this receptor 'superfamily' that have been identified through molecular approaches, but for which there is no known ligand, hence their designation as 'orphan' receptors [74, 77].

Historical perspectives

In 1959, Jensen and Jacobsen [1] successfully synthesized [^{3}H]estradiol-17β, and injected this radioactive tracer into

Table 2. Examples of nuclear transcription factor receptors for lipophobic substances.

Steroid hormone receptors
- estrogen receptor (ER), α and β
- progesterone receptor (PR), A and B
- glucocorticoid receptor (GR), α and β
- mineralocorticoid receptor (MR)
- androgen receptor (AR)
- bile acid receptor (FXR)

Secosteroid receptors
- Vitamin D receptor (VDR)

Iodo-thyronine receptors
- thyroid hormone receptors (TR), α and β

Retinoid receptors
- retinoic acid receptor (RAR), α, β and γ
- retinoid X receptor (RXR), α, β and γ

Fatty acid receptors
- peroxisome proliferation activating receptor (PPAR), α, β and γ

Orphan receptors
- ROR, α and β
- COUP-TF
- HNF-4
- constitutive androstane receptor (CAR)*

[74–78].
* CAR is no longer an orphan, but differs from the action of the other known receptors in that ligand binding to CAR turns off constitutive activation of gene expression [78].

female rats and observed prolonged retention of the radiolabeled estrogen in its reproductive target tissues. This observation provided the first direct evidence for the previously hypothesized 'receptors' for estrogen in this system. Moreover, similar experiments have been used in many steroid-responsive systems, including the $1,25(OH)_2D_3$ hormonal system [68], to identify target tissues and/or provide evidence for receptor-directed mechanisms.

In all of these systems, [^{3}H]-ligand binding assays were then used to identify specific and high-affinity binding characteristics expected of a specific 'receptor' protein [79, 80], putative receptor sizes (which vary from 55 to 120 kDa), apparent cytoplasm-to-nuclear translocation and subunit association/dissociation [81, 82]. In particular, many of the ligand-binding receptor subunits (table 3) were shown to associate with several heat shock proteins (HSPs) in a large holoreceptor complex (~300 kDa) in the unliganded state, to dissociate from the HSPs upon hormone binding and to then dimerize with another ligand-binding nuclear receptor before initiating their biological activity. In some cases the dimer partner is a form of the same receptor, forming a homodimer (e.g. ER), but in other cases the receptor heterodimerizes (e.g. VDR) with a dissimilar receptor, most often the promiscuous heterodimer partner RXR (table 3).

In parallel to studies of receptor binding sites, many of the molecular tools used today were developed specifically to generate information on the steroid/thyroid receptor mechanisms, including analyses of RNA synthesis, isolation and generation of complementary DNA strand (cDNA) to deduce amino acid sequence in the corresponding protein. The next steps were isolating and sequencing the relevant gene segments and then identifying promoter sequences 5′ to the coding sequence [73]. It is impossible to overstate the importance of these contributions, nor the amount of work (and often tedium) that they entailed.

Early studies of the intracellular location of steroid, but not thyroid, receptors led to the conclusion that they were located in the cytoplasm in the unliganded state, but were 'activated' upon hormone binding to a form with high affinity for nuclei/chromatin/DNA, resulting in nuclear 'translocation' of the receptor dimer (reviewed in [1]). However, in the early 1980s, evidence from a number of different experimental approaches, including autoradiography, subcellular fractionation and enucleation, and immuno/histocytochemistry, indicated that most of the members of this receptor family are nuclear proteins even prior to ligand binding [1]. The apparent exception is the glucocorticoid receptor [88].

Nuclear receptor structure

When the first steroid receptors were cloned in the mid-1980s, details about their functional regions began to be elucidated [88–91]. As shown in figure 1, the receptors are modular proteins, with an NH_2-terminal regulatory region (activation function-1 or AF-1), a midregion DNA binding domain, and a COOH-terminal region in which the ligand binding and AF-2 regions overlap [70, 84, 92, 93]. The length of the N-terminal domain is the most vari-

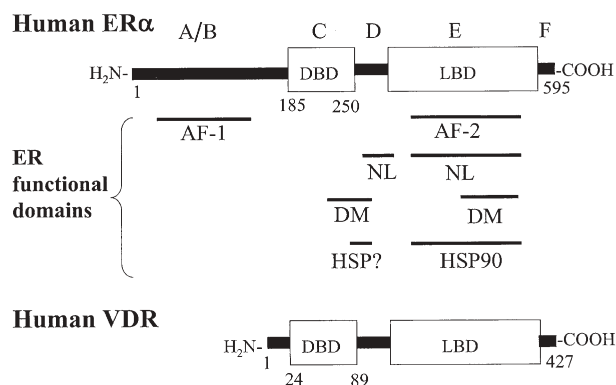

Figure 1. Functional domains of the human ER and VDR. The diagrams show the locations of the principal structural domains A–F with the amino acid residues numbered from -NH2 to -COOH termini. DBD, DNA binding domain; LBD, ligand binding domain; AF-1 and AF-2, activation functions 1 and 2; NL, nuclear localization sequence; DM, dimerization sequence; HSP90, heat shock protein 90 [70, 84, 92, 93].

Table 3. Differences in receptor subunit location and dimer partners: class I vs class II nuclear receptors.

	Unliganded receptor		Principal activated receptor form	
	Location	HSP complex?	Homodimer	Heterodimer
Class I				
ER	nucleus	+	+	–
PR	nucleus	+	+	–
GR	cytoplasm	+	+	–
Class II				
TR	nucleus/HRE	–	–	RXR
VDR	nucleus/HRE	–	–	RXR
RAR	nucleus/HRE	–	–	RXR
RXR	nucleus/HRE	–	–	multiple partners

HRE, hormone response element; HSP, heat shock protein (sSee table 2 for other definitions). [1, 73, 76, 83–87].

able property of the members of the nuclear receptor superfamily, ranging from significant length, for example in the ER, to being essentially absent, as in the VDR. Thus, the AF-1 and AF-2 regions play different roles in different receptors and under different physiological conditions. Much of this difference also results from the fact that AF-1 and AF-2 also contain recognition sequences for the transcriptional cofactors [94] described below. Specific sequences in each receptor also mediate other functions, such as binding to HSP90 and nuclear localization. In addition, each receptor has a number of potential phosphorylation sites which are now thought to be sites of regulation (up or down) of hormone responsiveness, with different intracellular signals (for example MAP kinase, PKC and PKA), targeting different phosphorylation sites within each receptor protein [95, 96]. While there is much yet to be done in understanding these patterns of regulation for each receptor, it is clear that there is complex potential for regulating the level of function for each receptor species.

As the initial receptors were cloned, the close evolutionary relationships between receptors for rather different ligand species, for example thyroid hormone vs vitamin D vs steroid hormones, became apparent. Moreover, the receptor 'superfamily' expanded even further when the first 'orphan' receptor, COUP-TF, was discovered, followed quickly by numerous other orphans [74, 77]. And while ligands have not yet been identified for most orphan receptors, the level of thought on the range of substances that can bind to and activate these receptors (table 2) has now evolved to the point that investigators understand that many non-steroidal substances may be candidates for activating ligands for this family of proteins.

The search for additional members of this receptor family has also led to finding multiple forms of many of the receptors (table 2). Early biochemical studies demonstrated the presence of two PR forms, PR-A and PR-B [81], which have now been shown to be generated from different start sites of a single PR gene [97]. On the other hand, the more recently distinguished ERα and ERβ are expressed from different genes [76]. Moreover, although different VDR forms have been hypothesized from biochemical studies [98], molecular studies have not provided evidence for multiple VDR forms.

Although early biochemical studies suggested that the nuclear receptors could bind DNA, early interpretations of the receptor structures deduced from their amino acid sequences yielded the initially surprising interpretation that each receptor contains two 'Zn fingers' (a protein loop anchored by Zn^{++} in a complex with either four cystine residues or two cystine and two histidine residues) in what is now termed the DNA binding domain [99]. Since Zn fingers are a major motif by which transcription factors bind to DNA [100], this observation confirmed the long-held suspicion that the steroid receptors function as nuclear transcription factors.

Table 4. Preferred HRE sequences/structures for the nuclear transcription factor receptors.

Receptors	Consensus HRE sequence	Spacer length (nt)
PR/GR/MR/AR	GGTACAn_3TGTTCT	3
ERE	GGTCAn_3TGACC	IR3
TR	AGGTCAn_xAGGTCA	DR2,DR4
VDR	AGGTCAn_xAGGTCA	DR3
RAR	AGGTCAn_xAGGTCA	DR2, DR5, DR7
RXR	AGGTCAn_xAGGTCA	DR2

nt, nucleotides; DR, direct repeat; n_x, variable number of nucleotides; IR, indirect repeat. [73,77,84,101].

Members of the steroid receptor superfamily function as nuclear transcription factors

Since the early biochemical studies characterizing the steroid receptors, it has been apparent that they are DNA-binding proteine. However, studies of the specific DNA sequences recognized by the receptors initially provided unexpected results. From the very different physiological/biochemical effects of the different hormones and their receptors, investigators expected that the DNA-binding sequences would be very different between the receptor forms. Instead, there is substantial similarity/identity between the preferred nucleotide sequences recognized by many of the receptors (table 4). The main difference distinguishing many of the the DNA binding sites for specific receptor species is the number of 'spacer' nucleotides between two half-site binding sequences [102]. These DNA sequences recognized by the receptors are termed hormone response elements (HREs) and are inducible enhancer elements [73]. They mediate receptor-activated transcriptional control irrespective of their location within the promoter region and independent of their orientation. The HREs are usually 5–6 bp sequences (table 4), arranged in pairs (half-sites) as a symmetrically repeated pattern, usually as a direct repeat (DR) or an inverted repeat (IR), although other sequence relationships have been described [73, 77, 101, 102]. Each partner in the receptor dimer binds to one of the half-site sequences. The nucleotide sequence reported for these receptors is a 'consensus sequence' in that not all HREs represent this exact sequence, but rather are often slight variations of these sequences. More than likely, there is regulatory information embedded in these small sequence changes that we do not yet understand. In between these specific 5–6 bp half-sites is a stretch of nucleotides whose sequence is not conserved and is thought to be unimportant, but which function as 'spacers' between the two half-sites. Thus, what is important about the spacer region is the number of nucleotides, that is the distance between the half-sites [102]. Undoubtedly, this distance de-

fines the size/shape of the receptor dimer pair that can fit onto the DNA helix and still bind to the HRE half-sites. The preferred spacing for the different receptor species that share the same HRE sequence is defined as DRX, where X is the length of the spacer, or sometimes as IRX. And the spacer length usually varies from 1 to 5 nucleotides (nt), although spacing may vary from 0 to 9 nt (table 4).

Nuclear receptor/cofactor interaction

Similar to other transcription factors, these nuclear receptors do not act alone in regulating transcription. Instead, there are numerous cofactors (coactivators, corepressors and mediators) that interact with the receptor/HRE complex and mediate communication to the transcriptional complex. The first indication that these are cofactors derived from evidence that overexpression of two or more receptors inhibited ('squelched') transcription from any single receptor, apparently by competing for a limited pool of transcriptional cofactors [103]. Discovery of receptor/coactivator interactions began with the identification of SRC-1 (steroid receptor coactivator-1) [104], one of a family of p160 factors that act as coactivators in different complexes and under different conditions. The main steroid receptor coactivator complex seems to consist, at a minimum, of one of the p160 proteins (e.g. SRC-1, TIF-2, GRIP), p300/CBP (a universal coactivator/cointegrator for transcriptional complexes) and the PCAF complex (reviewed in [105–108]). When the activated receptor dimer binds the HRE, this coactivator complex is recruited to the site. Its association with the receptor complex is apparently very transient, so it is rather difficult to study the entire receptor/coactivator complex. The coactivators play important roles in remodeling the nucleosome structure of the chromatin, which is an essential step in opening up the promoter site to allow transcriptional activation [109, 110]. Most members of the coactivator complex exhibit histone acetylase (HAT) activity [105–108], and some of these factors are also acetylated [109]. These activities are important because histone acetylation allows unraveling of the tightly coiled histone/DNA complex. Other enzymatic activities, such as methylase and helicase activities [110, 111], may contribute to this process.

Another set of cofactors (e.g. N-COR, SMRT) function as corepressors in these systems [105–108,112]. Most notably, the corepressors associate with the unliganded class II (table 3) receptor heterodimer complexes on the HRE sites, and prevent transcriptional activation in the absence of the respective ligands. In part, corepression is achieved by recruiting histone deacetylase (HDAC) complexes to ensure tight packaging of the histone/DNA complex [113, 114]. Thus, the unligarded TR/RXR or VDR/RXR dimers sit on their HRE elements, held in a transcriptionally inactive state by a corepressor complex. Upon ligand binding to the receptor, the corepressor complex dissociates, and the coactivator complex is recruited [108]. Interestingly, in some cases, antagonist-bound ER complexes also recruit corepressors [115], accounting in part for the inability of antagonist-bound receptors to activate transcription.

After the coactivator complexes assist in chromatin remodeling and dissociate from the receptor complex, another protein complex is recruited. These 'mediator' complexes, termed for example DRIP (D receptor interacting proteins) or TRAP (TR associated proteins), consist of 9–14 proteins which are highly conserved in eukaryotic systems and appear to function as the final step in activating the polymerase activity of the transcriptional complex [108, 116, 117].

While it is not yet possible to look at the entire receptor protein, different conformations of the receptor LBDs (with and without ligand) have been studied by X-ray crystallography with much similarity found between the LBDs of different receptor species (reviewed in [117]). As illustrated schematically in figure 2A, each LBD has 12 α-helix regions (H1–H12) that fold in three-dimensional structures to define essential functional domains, including in particular the ligand binding pocket and the regions of AF-2 that bind the coactivator complex. While ligand binding alters the locations of several of the helices, the movement of H12 is the most remarkable [118]. H12 is the most -COOH terminal of the LBD α-helices and contains the core residues critical to the AF-2 function. In the unliganded state, H12 is oriented away from the structure encompassed by H1–H11 (fig. 2A). When an activating ligand is bound (fig. 2B), H12 folds up across the ligand binding pocket. But when an antagonist binds, H12 flips away from the LBP and lies over the coactivator binding site on H3 (fig. 2C). Of course, the actual details are much more complex than briefly described here; full details are available elsewhere [118].

Pleiotropic effects of steroid/lipophilic hormones

In the course of studying hormonal effects in any endocrine system, most initial studies focus on actions and receptors in principal target tissues, which most often means those targets where the hormone exerts readily observed/measured effects. In the estrogen response system, these were the same tissues studied by Jensen and Jacobson, that is the uterus and vagina [1]. However, in all these systems, as investigators began to understand more about hormone action and as they developed better receptor assays, it has become clear that many other tissues are targets for hormone action. Thus, in the area of estrogen biology, receptors and actions have been discovered in a number of unanticipated locations [119], including for example the liver, which served as a control nontarget

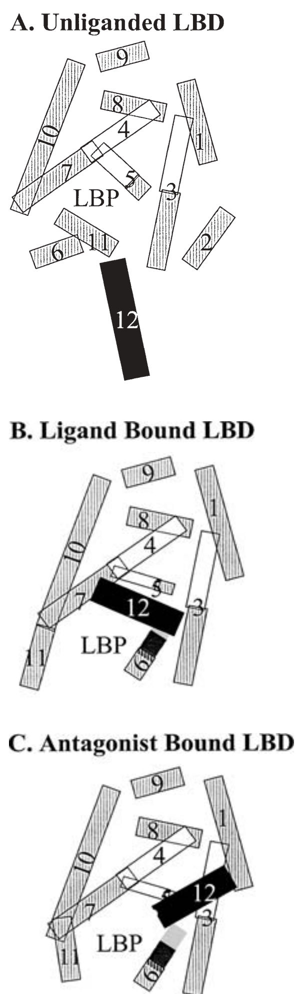

Figure 2. Schematic summary of the X-ray crystallographic analysis of conformations of α helices 1–12 (H1–H12) of the ligand binding domain (LBD) of retinoid receptors: comparison of conformations of the unliganded RXR (*A*), ligand bound RAR (*B*) and antagonist-bound RAR (*C*) forms. The open bars represent the coactivator/corepressor binding site; the dark bar represents the most distal helix of the LBD, helix 12, which is also the core domain of AF-2. LBP, the ligand or antagonist binding pocket. The movement of helix 12 demonstrates the open nature of the LBP in the unliganded state (*A*), its positioning over the ligand/LBP region when ligand is bound (*B*) and its interference with the coactivator binding surface in the presence of antagonist (*C*). Adapted from Bourguet, Germain and Gronemeyer [118].

tissue in the early studies. The story is perhaps even more remarkable in the vitamin D endocrine system. Early studies focused on the anticipated actions of this hormonal system in regulating plasma Ca^{++} and PO_4^- homeostasis, principally through actions on kidney and intestine [68]. However, subsequent studies led to the discovery of VDR and actions in most tissues of the body (reviewed in [120, 121]). While some of these effects relate to processes that regulate the vitamin D endocrine system, probably the most surprising observations were those that established effects of $1,25(OH)_2D_3$ on growth and differentiation of numerous tissues, including for example hematopoietic cells, breast, prostate, skin and muscle [120, 121]. From these unanticipated effects, there is substantiated interest in developing noncalcemic analogs of $1,25(OH)_2D_3$, due in part to its inhibitory effects on growth of some cancers, for example breast and prostate.

Ligand-independent nuclear receptor activation

Another unanticipated observation in the area of mechanisms of action of the nuclear transcription factor receptors began when dopamine was demonstrated to activate, but not bind to, the orphan receptor COUP-TF [122]. Subsequent studies have demonstrated that dopamine, acting through the dopamine D1 receptor, can ligand independently activate many of the nuclear transcription factor receptors [123]. While the mechanisms are not well defined, it is likely that signal transduction pathways activated by dopamine (and some other membrane-active ligands, including growth factors) induce specific phosphorylations on either the nuclear receptors or their coactivator partners [123–126]. The latter mechanism was observed when phosphorylation of SRC-1 was shown to be a mechanism of ligand-independent activation of the PR by cAMP [127]. Moreover, these ligand-independent regulatory pathways are specific with respect to the membrane-active agents, the nuclear receptors, and the tissue and physiological context.

Conclusions

The field of steroid hormone receptors has evolved rapidly in the last few decades. From early models in which simple mechanisms were proposed to account for complex responses, we are now beginning to appreciate greatly more complicated mechanisms leading to complex responses. The combined pleiotropic actions of membrane and nuclear receptors, in a coordinated fashion, are more likely to explain the orchestration of rapid nutrient uptake, vesicular trafficking and gene regulation than either pathway alone (fig. 3). Numerous reports and reviews have discussed the plethora of signaling pathways initiated by estrogen and $1,25(OH)_2D_3$ [26, 37, 40, 128–130]. These include protein kinase A, PKC, PI-3 kinase and extracellular receptor kinase, among others. As schematically presented in figure 3, these signaling cascades are capable of activating rapid responses, as well as gene transcription. In the case of $1,25(OH)_2D_3$, an additional level of complexity is added by the fact that the $1,25D_3$-MARRS protein translocates to the nucleus upon ligand binding [39], where it is possible that it too may interact with transcription factors. Finally, the most striking similarities between membrane-initiated actions for both estrogen and $1,25(OH)_2D_3$ appears to be the involvement

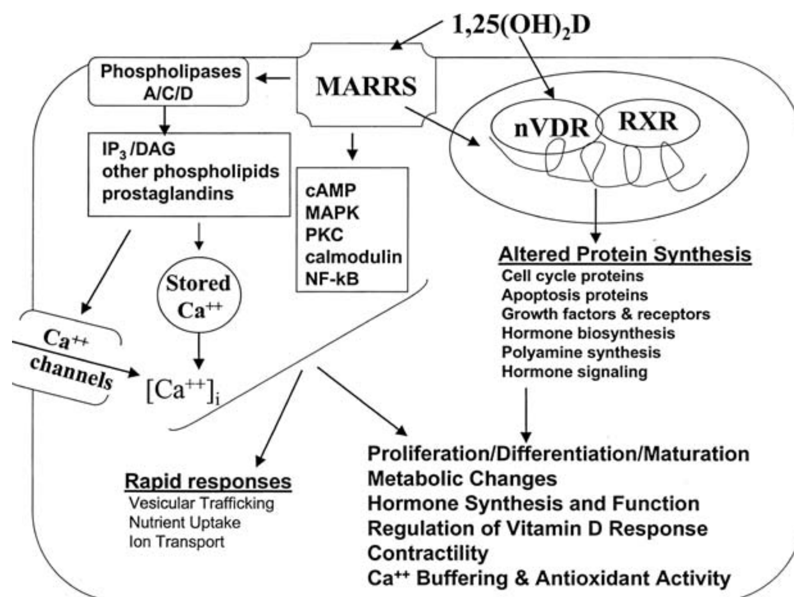

Figure 3. Generalized model of the membrane and nuclear mechanisms of action of 1,25$(OH)_2D_3$. (Left side) 1,25(OH)2D_3/MARRS interaction alters signal transduction pathways, including changes in intracellular Ca++, PKC, phospholipase-dependent processes and NF-kB. 1,25$(OH)_2D_3$-MARRS binding also results in nuclear translocation of MARRS. (Right side) 1,25$(OH)_2D_3$/VDR interaction results in altered gene expression and changes in mRNA and protein synthesis. Collectively, these complex pathways induce pleiotropic responses specific to each cell affected.

of at least two different 'receptors', the classical ones, as well as ones in the 'endoplasmic reticulum protein' family. It is likely that such parallel receptor systems will exist for other steroid hormones.

Acknowledgments. This work was supported in part by NRI-CSREES-USDA grant 2004-35206-14134 (I. N.), by the Louisiana Board of Regents Millenium Trust Health Excellence Fund (M. W.) and by an NSF Advance Leadership Award (M. W.)

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