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Hormone response element binding proteins: novel regulators of vitamin D and estrogen signaling

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Abstract

Insights from vitamin D-resistant New World primates and their human homologues as models of natural and pathological insensitivity to sterol/steroid action have uncovered a family of novel intracellular vitamin D and estrogen regulatory proteins involved in hormone action. The proteins, known as “vitamin D or estrogen response element-binding proteins”, behave as potent *cis*-acting, transdominant regulators to inhibit steroid receptor binding to DNA response elements and is responsible for vitamin D and estrogen resistances. This set of interactors belongs to the heterogeneous nuclear ribonucleoprotein (hnRNP) family of previously known pre-mRNA-interacting proteins. This review provides new insights into the mechanism by which these novel regulators of signaling and metabolism can act to regulate responses to vitamin D and estrogen. In addition the review also describes other molecules that are known to influence nuclear receptor signaling through interaction with hormone response elements.

I. Introduction

Target cell responses to steroid/sterol hormones rely on a variety of factors including the expression and regulation of cognate nuclear receptors (1-3), their associated accessory proteins (4) and pre-receptor regulation of the actual hormone ligands (5-6). In previous studies of New World primates (NWP) and Old World primates (OWP), we identified a novel class of proteins involved in regulating vitamin D and estrogen signaling at the level of gene transcription (7). NWP characteristically exhibit target organ resistance to steroid hormones such as estrogens, in part, because of the nutritional and environmental ecology of NWP (i.e. dietary exposure to high levels of plant phytoestrogens, and elevated levels of vitamin D as a consequence of routine exposure to UVB light). Previous work by our group has shown that steroid/sterol hormone resistance in NWP involves over-expression of response element-binding proteins (REBiPs) that compete with nuclear receptors for binding to *cis* regulatory elements in target DNA (8-9). In OWP, the vitamin D response element-binding protein (VDRE-BP) occupies target gene vitamin D response elements (VDREs) in the absence of significant levels of hormonal 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D], the biologically active form of hormone. However, when levels of 1,25(OH)₂D increase, the liganded vitamin D receptor (VDR) is then able to displace the VDRE-BP and induce target

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gene transcription. In NHPs, an over abundance of VDRE-BP means that much higher levels of $1,25(\text{OH})_2\text{D}$ are required to induce VDR signaling (10). In humans, normal response to $1,25(\text{OH})_2\text{D}$ also involves competition between the VDR and VDRE-BP (11), and excess VDRE-BP expression has been described for a patient with hereditary vitamin D resistant rickets (HVDRR) (11-12). These studies also revealed that the human VDRE-BP is identical to heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2 (11), a member of the hnRNP family of RNA-interacting proteins.

Lastly, NHPs also involve novel regulatory features of one of the receptors for estrogen (ER α), implicated in hormone resistance which involves both the estrogen response element binding protein (ERE-BP) (13-16). This review will briefly cover the molecular mechanisms involved in hormone resistance, and discusses the possible significance of the various modes for the physiologic actions of vitamin D and estrogen hormone receptors.

II. Nuclear receptor superfamily of steroid hormone receptors and gene activation

Nuclear receptors (NRs) comprise a class of transcription factors and signaling molecules in vertebrates. The NR members include receptors for hydrophobic molecules such as steroid/sterol hormones (e.g. estrogens, glucocorticoids, progesterone, mineralocorticoids, androgens, vitamin D₃, ecdysone, oxysterols and bile acids), retinoic acids (all-*trans* and 9-*cis* isoforms), thyroid hormones, fatty acids, leukotrienes and prostaglandins (17-18). NRs mediate gene transcription of target genes by binding to different response elements and forming complexes comprising of co-regulatory proteins to affect chromatin remodeling and epigenetic modifications often at locations distant from the transcription start site. The NRs for all classes of steroid hormones can control gene transcription either by activating transcription factors or by acting as transcription factors altogether. Classically, NRs function in three key steps: repression, derepression and transcription activation (18-19). Repression involves recruitment of a co-repressor complex with histone deacetylase activity (HDAC). That is, eukaryotic transcription is impaired by a repressive chromatin environment of the regulatory regions of genes. Derepression occurs after ligand binding, which dissociates this “repressed” complex and attracts a first co-activator complex, with histone acetyltransferase (HAT) activity, resulting in chromatin decondensation assumed necessary but not sufficient for activation of the target gene. In the third step, the HAT complex dissociates and a second co-activator complex is formed (e.g. TRAP/DRIP/GRIP/ARC), which is able to establish associations with the basal transcription machinery, and thus results in transcription activation of the target gene. It should also be noted that this mechanism is not general, since some NRs may act as activators independently of a ligand, whereas others are unable to interact with the target gene promoter in the absence of ligand, and not to mention the variety in co-regulatory complexes.

Steroid/sterol receptors are evolutionarily conserved ligand-dependent and -independent transcription factors that belong to the diverse NR superfamily of proteins (17-18). These receptors can be found at the plasma membrane, in the cytosol and also in the nucleus of target cells. The cell membrane-crossing lipophilic hormones for steroid receptors are based on diverse chemical structures of the steroid nucleus, although several receptors are capable of binding nutritional lipids as well. Receptors that associate with sterol ligands in vertebrates are the main focus of this review and include: 1] PXR, which binds pregnenolone; 2] LXR, that recognizes 22-hydroxycholesterol; 3] FXR, which binds bile acids such as chenodeoxycholic acid; 4] CAR, that associates with androgen metabolites like androstenol; and 5] VDR, the receptor for the renal $1,25(\text{OH})_2\text{D}$ hormone that stimulates intestinal calcium absorption and bone remodeling (Table 1).

NRs share a common canonical structural organization. Structural domains found to be responsible for ligand binding, for DNA and co-regulator binding, receptor dimerization, and transactivation are highly conserved across the family (20). The N-terminal region (A/B domain) is highly variable, and features at least one constitutively active transactivation region (AF-1) and several autonomous transactivation domains (AD) (21). The most conserved region is the DNA-binding domain (DBD, C domain), which notably contains two “zinc finger” domains and the P-box, a short motif responsible for DNA-binding specificity on target sequences typically containing a six base pair AGGTCA-like motif, and is involved in homo- or hetero-dimerization of nuclear receptors. The hinge region (D domain) is thought to be a flexible domain that connects the DBD with the moderately conserved in sequence ligand binding domain (E domain), and influences intracellular trafficking and subcellular distribution.

The ability of ligand-bound sterol receptors such as the VDR to transactivate a sterol-responsive gene depends on the presence of AF1- and AF2-interacting “bridging” nucleoproteins, and the co-regulators that have chromatin-remodeling and other enzymatic activities (22) (Figure 1). The co-activator can enhance transcription initiation by stabilizing the assembly of the RNA polymerase holoenzyme enabling faster clearance of the promoter. Co-activators may control many other substeps of transcription, including elongation, RNA splicing, and termination and degradation of the co-activator complex. The known co-activators of NRs belong to several families. The nuclear receptor co-activators 1-3 (*NCOA1-3*) are transcriptional co-regulatory proteins which harbor several nuclear receptor interacting domains and intrinsic histone acetyltransferase activity. NCOAs are recruited to DNA promoter sites by ligand activated nuclear receptors and, in turn, acetylate histones. The p160 family and the riboprotein co-activator steroid receptor activator (SRA) include members whose activities are constrained to nuclear receptors and transcription factors are known to interact with a member of the transcriptional enhancer factor (TEF) family of transcription factors (23) and covalently modify histone tails (24). The CREB-binding protein (CBP)/p300 family of co-activators and the CBP/p300-associated PCAF are crucial for other signal transduction systems as well, including the protein kinase A-cAMP-CREB, the growth factor-cfos/cjun, the growth factor/cytokine Jak-STAT, and the cytokine-NFκB pathways. Because of their broad functions, CBP and p300 have been also called “co-integrators”. Lastly, other posttranslational modifications, such as serine phosphorylation, are necessary to make sterol/steroid receptors transcriptionally active.

III. Hormone resistance

Occasionally, hormones are unable to exert their specific responses in certain tissues due to resistance to a particular hormone. In most cases hormone resistance is caused by mutations in hormone receptors affecting critical domains involved in hormone binding, receptor dimerization, and transactivation or by functional alterations of hormone receptor interactors, such as serum binding proteins, to disturb various signaling pathways. Hormone resistance syndromes have been investigated according to receptor types to illustrate the diversity of hormone resistance or disorders. These receptor types include seven transmembrane G-protein-coupled receptors (24-26), tyrosine kinase receptors (27), cytokine family of receptors (28), and transforming growth factor beta (TGFβ) serine kinase group of receptors (29). Also, G-protein mutations (e.g. *GNAS1*) cause Albright's hereditary osteodystrophy (30), which leads to intramembranous ossifications progressively developed from the dermis.

As might be expected, a variety of resistance syndromes involve members of the NR superfamily, including resistance to androgens, vitamin D₃, thyroid hormone, glucocorticoids and estrogen (Table 1). Many NRs share multiple isoforms. For example,

both the ER and liver X receptor (LXR) are encoded by α and β genes (Table 1). Consequently, mutations that eliminate one receptor isoform may not completely eliminate hormone action, or may even cause gain of function of the non-mutated isoform. In addition, mutations in orphan NRs such as DAX-1 (*NR0B1*) result in compromised glandular development, thereby indirectly causing hormone resistance resulting in both X-linked congenital adrenal hypoplasia and hypogonadotropic hypogonadism (31). DAX-1 lacks the normal DNA-binding domain contained in other NRs and acts as a dominant-negative regulator of transcription of other NRs including steroidogenic factor 1. This protein also functions as an anti-testis gene by acting antagonistically to the transcription factor SRY (Sex-determining Region Y) (32).

In other instances of hormone resistance, processing variations may lead to differences in the activity of a hormone at the “pre-receptor” level. For example, an endogenous antibody to a hormone may inactivate the hormone and create resistance of a pre-receptor nature. Furthermore, auto antibodies generated against a polypeptide hormone receptor may act as an agonist or as an antagonist to dictate the action of the polypeptide ligand resulting in Grave's disease, anemia, and myasthenia gravis as examples (33). Another form of resistance, sometimes referred to as “post-receptor” resistance, is typified by acquired forms of insulin or leptin resistance, and the pathophysiology remains less than well characterized (34-35). A post-receptor defect generates a proportionate reduction in hormone action at all hormone levels, including maximally effective concentrations, and this is termed a decrease in hormone responsiveness in the face of normal insulin receptor binding. This suggests a defective coupling of NR binding to the subsequent steps of hormone action. Lastly, artifactual hormone resistance can be due to patient medication non-compliance, formulation, absorption and metabolism when increasing hormone treatment is without apparent effects.

IV. Hormone response element (HRE) interactors

DNA contains conformational and topological signatures that are implemented in chromatin and that controls the accessibility to *cis*-acting elements (25-26). NR-induced nucleosomal changes influence the receptor-mediated recruitment of chromatin remodeling factors as part of steroid hormone signaling responses. Generally speaking, these interactions can be physiologically modulated by cell and promoter context-specific factors to facilitate specific and optimal responsiveness of gene expression to hormone stimulation.

A unique mode of regulation, one that we postulated in the 1980s based on observations made in NHPs with regard to steroid hormone signaling (27-28), involves the functional antagonism between hormone receptors in play and other non-targeting hormone receptors or non-receptor *cis*-acting transcription interactors at the designated hormone response elements (HREs). This mode of regulation has been expanded and encompasses not only VDREs and EREs, but also ROR α REs, LXREs, (decoy) GREs, AREs, steroidogenic consensus half-sites and non-classical composite HREs (Table 2). Furthermore, in addition to the findings of our group, the specific VDRE and ERE regulatory control by ‘non-receptor’ response element binding proteins has been endorsed by the involvement of other non-related components such as the ubiquitous transcription regulator YY1 (i.e. Ying Yang 1, a GLI-Kruppel class of zinc finger protein) (29) and the scaffold attachment factor B (30), respectively. These findings provide additional evidence for the complex regulation of steroid hormones and their receptors targeting VDREs and EREs in different cellular systems.

In osteoblasts the functional VDRE-containing osteocalcin (OC) gene is transcriptionally active within mature post-proliferative cells at the onset of matrix mineralization to form

bone. Guo et al. (29) sought to understand the vitamin D-dependent modulation of OC gene transcription in terms of better defining the interrelationships between the VDR and the non-steroid hormone-related transcription factor YY1. YY1 is involved in either repressing or activating a diverse number of promoters through recruitment of either histone deacetylases or acetyltransferases, thus implicating histone modification at or near VDREs to promote chromatin remodeling (31). The authors found that the multifunctional YY1 regulator suppressed the normal $1,25(\text{OH})_2\text{D}$ -induced transactivation of the OC gene by competing for the VDR-RXR heterodimers for VDRE binding (i.e. much like the hnRNP proteins; see later), and also interfered with the protein-protein interaction between DNA-bound VDR-RXR and transcription initiation factor TFIIB. Furthermore, the authors showed that YY1 was present at constitutive levels in proliferating osteoblasts when VDR-RXR heterodimer levels were low, a possible mechanism by which YY1 associates with the OC-VDRE to prevent hormone receptor DNA interaction to block premature vitamin D-dependent transactivation reserved for mature cells. Previous reports suggest that both hnRNP and YY1 proteins are key functional modulators of vitamin D responsiveness in the *OC* and *CYP24A1* genes. Our recent unpublished data suggests that hnRNP proteins can modulate VDR actions of several other genes related to bone cellular turnover and homeostasis (Table 2). Both YY1 and hnRNP appear to be components of regulatory switches that control bone-specific genes. In both cases, elucidating the molecular mechanisms mediating steroid hormone regulation of skeletal development will help to clarify the basis of regulatory perturbations associated with skeletal pathologies such as hereditary vitamin D resistant rickets (HVDRR).

Although there are other examples of competition between NRs and non-receptor transcription factors for DNA binding, as yet there are no reports of direct HRE interactors that disrupt native hormone signaling for steroid nuclear receptors PXR, CAR, ERR, MR, and PR. There are reports describing a class of composite hormone response elements (CHREs) in which the NR functions amongst other regulators and enhances or represses transcription (32). That this regulation of CHREs requires the combined actions of the target receptor together with one or more non-receptor (overlapping) sequence-specific regulator provides another level of complexity (33). Such loci can designate either hormone-dependent enhancement or repression of transcription, and in the absence of the appropriate non-receptor factor, fail altogether to confer hormonal regulation, despite the presence of a functional receptor. This is different from simple, more common consensus HREs where the nuclear receptor functions independently of other regulators to enhance transcription (34), suggesting the receptor activation domains are positioned close to promoters. In the case of the plfG composite element (i.e. GRE-AP-1), the involvement of a partial AP-1 sequence and subunit composition of AP-1 (i.e. c-Jun homodimers vs. c-Jun/c-Fos heterodimers) determines the nature of the hormone response mediated by the GR (35). Together, these findings imply that sequence positioning and ternary complexes of receptor and AP-1 at plfG determine the direction and magnitude of the subsequent hormone response. Thus, the plfG sequence represents a well-delineated composite element that mediates functional interactions of sequence-specific regulators from two well-characterized factor families.

Another class of proteins that may act as competitors for NR-DNA interaction are known non-target (orphan) nuclear receptors that have the intrinsic ability to regulate the expression of specific gene networks through competition between endogenous native receptors for the same recognition site (36). For example, Rev-ErbA beta (NR1D2) recognizes a HRE with specificity similar to that of ROR α and does not activate transcription alone, but rather acts as a competitive repressor of ROR α function (36). On a similar note, there is cross-talk between the T3 and LXR response elements, which have similar configurations, for the respective thyroid hormone receptor (TR) and LXR α affecting signal transduction pathways (37). Specifically, the major isoform of TR in the liver, TR- β 1, binds directly to LXREs

located in the promoter of the sterol regulatory element binding protein 1c and interacts with co-repressors important to the unliganded TR-mediated suppression of LXR α -transactivation to regulate lipid metabolism.

Recently, the farnesoid X receptor (FXR), important in bile acid homeostasis, was identified as a negative modulator of the androgen-estrogen-converting aromatase enzyme in human breast cancer cells (38). Mechanistically in another report, mutagenesis studies, electrophoretic mobility shift, and chromatin immunoprecipitation (ChIP) analysis utilizing Leydig tumor cells reveal that FXR was able to compete with steroidogenic factor 1 (SF-1), a key regulator of reproduction, in binding to a common sequence present in the aromatase promoter region interfering negatively with its activity (39). In a similar fashion, heat shock protein 27 (hsp27) enhances both growth and drug resistance in breast cancer cells, and is a bad prognostic factor in certain subsets of breast cancer patients. The hsp27 promoter contains an imperfect estrogen response element (ERE) that is separated by a 13-bp spacer that contains a TATA box to which the scaffold attachment factor B (SAF-B) binds (30). Scaffold attachment factors are a subset of nuclear matrix proteins (NMP) that interact with matrix attachment regions. Over expression of SAF-B results in dose-dependent decreases in hsp27 promoter activity suggesting this non-nuclear receptor protein as a mediator of breast cancer development at the level of hormone response element binding.

Non-coding RNAs (ncRNAs) are functional RNA molecules that are not translated into a protein. Growth arrest-specific 5 (GAS5) is abundant in cells whose growth has been arrested due to serum or nutrient starvation. Interestingly, the GAS5 ncRNA has the ability to bind to the DNA-binding domain of glucocorticoid receptors (GRs) by acting as a decoy GR response element (GRE), thus competing with promoter DNA GREs for binding to the GR to mediate several downstream responsive genes such as inhibitor of apoptosis 2 (40). Glucocorticoids are known to influence gene transcription and have diverse activities on immune response, cell growth, energy expenditure, and survival, whereby presence of decoy GAS5 ncRNA has the ability to modulate the transcriptional read-out of available GR.

Lastly, another interactor which has the potential to disrupt NR interaction with HREs include small molecule polyamides designed to compete with NRs for binding to HREs (41-42). Polyamides affect the DNA-dependent pathway selectively by interfering with the protein-DNA interface. These oligomers can occur both naturally and artificially, achieving sequence specificity via side-by-side pairings of the heterocyclic amino acids to the minor groove of DNA. For example, a DNA-binding polyamide targeted the consensus GRE of the glucocorticoid-induced zipper (GILZ) gene to block its expression as well as other known GR target genes assessed in genome-wide expression analysis (41). Furthermore, the same class of polyamide compounds was recently shown to regulate prostate cancer by binding and suppressing the consensus androgen response element (ARE) and prostate-specific antigen gene within the physiological context to inhibit AR activity (42). On a low note, it is well known that many pesticides are able to inhibit or activate NR systems to affect levels of sex hormone to alter development or expression of the male and female reproductive systems (43), and based on recent findings this may also be applicable to readily utilized industrial polyamide resins alluded above.

As described above, in the literature there are several indications for interactions between the respective HREs and signaling molecules. The subject matter of HRE interactors is not generalized, with some receptors bound to the HRE even in the absence of hormone, sometimes functioning to suppress gene expression (44). The exciting observation that NRs seed formation of complexes at the HRE and comprise co-regulatory proteins and RNA has both greatly energized and further complicated our understanding of hormone action (45). The application of powerful tools to define the genome-wide location of NR binding sites

through methods such as ChIP followed by deep sequencing (ChIP-seq) has shown that receptors binds to chromatin throughout the genome, often far removed from putative transcription start sites, and may be found in intergenic regions, introns, and distal enhancers (46). These findings offer new avenues for potential future research and understanding of the complex mechanisms and particular target region(s) by which HRE interactors influence steroid hormone responses.

V. New World Primates and beyond co-regulators

NWPs such as marmosets, tamarins, and squirrel monkeys exhibit significantly higher circulating levels of steroid/sterol hormones than their OWP counterparts (including humans), but they are also profoundly “pansteroid/sterol” resistant to these hormones (47-48). In the early-to-mid 1980s we investigated an outbreak of rachitic bone disease in the NWP emperor tamarin (*Saguinus imperator*) colony at the Los Angeles Zoo (49-50). Analysis of epidermal cells isolated from these animal showed that the NWPs were protected against the potential adverse effects of sustained exposure to high circulating levels of hormones such as 1,25(OH)₂D and 17β-estradiol (E₂) as a consequence of elevated cellular expression of proteins that bind to VDR/ERα target gene promoter VDREs and EREs (10-12,15). Over abundance of these proteins, termed the VDRE- and ERE-binding proteins (VDRE-BP and ERE-BP, respectively) meant that much higher levels of 1,25(OH)₂D and E₂ were required to displace the chromatin-bound VDRE-BP and ERE-BP to promote receptor signaling. Purification, identification and characterization of the so-called VDRE-BP and ERE-BP as two distinct members of the hnRNP super-family, hnRNP C1/C2 and hnRNP C-like proteins, respectively, were accomplished in due time (10,15). Subsequent molecular cloning and over expression of these proteins and the identification of their human homologs showed them to be 1] dominant-negative inhibitors of 1,25(OH)₂D-VDR- and E₂-ERα-directed transcription (51) and to 2] interact with another category of intracellular chaperone proteins in the extended heat shock protein (hsp) family that bind both ligands and other regulatory proteins to fine tune transcriptional control over 1,25(OH)₂D- and E₂-driven genes in subhuman and human primates (16,52). We have termed the latter duo of hsp-related vitamin D and estrogen binding proteins as the intracellular vitamin D binding protein (constitutively expressed hsc70) and intracellular estrogen binding protein (hsp27), respectively. The remainder of the review describes in detail the observations that led to the discoveries of the HRE binding proteins and intracellular hormone binding proteins, and the subsequent work that incorporated these novel proteins into the vitamin D and estrogen signaling pathways.

VI. Vitamin D response element binding protein (VDRE-BP) and vitamin D resistance

The endocrine prohormones vitamin D₃, synthesized in the epidermis in response to UVB radiation, and dietary, plant-synthesized vitamin D₂ (ergocalciferol) are devoid of any biologic activity (53). Instead the hormonal activity of vitamin D is due primarily to the dihydroxylated metabolite 1,25(OH)₂D, a secosterol whose genomic mechanism of action is common to that of other steroid hormones, namely being mediated through binding to the VDR (53). In the liver, the enzyme vitamin D 25-hydroxylase catalyzes initial hydroxylation of vitamin D at carbon 25 to form 25-hydroxyvitamin D₂ or D₃ (25OHD), the major circulating form of vitamin D. In the kidney, another cytochrome P450 enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase), a product of the *CYP27b1* gene, catalyzes further hydroxylation of 25OHD into active, hormonal 1,25(OH)₂D. This form of vitamin D can then bind to the VDR and form a complex with the unliganded retinoid X receptor (RXR) in order to transactivate 1,25(OH)₂D-responsive genes via VDREs, thereby

enabling the regulation of physiologic events such as calcium homeostasis and cellular differentiation and proliferation (54).

Dysregulation of vitamin D metabolism and/or action can lead to defective bone mineralization as a result of intestinal malabsorption of calcium, hypocalcemia, secondary hyperparathyroidism, increased renal clearance of phosphorus and hypophosphatemia (Table 1). Endochondral bone formation is a two-step process: a chondrocyte template (cartilage anlage) is first shaped, in which osteoblasts then differentiate to form bone (55-56). Apart from the endocrine roles of vitamin D, chondrocytes and osteoblasts participate in autocrine and paracrine loops of vitamin D metabolism as they are able to convert 25OHD into 1,25(OH)₂D, suggesting that vitamin D plays a direct role in regulating their activities and endochondral bone formation (57-59) (Figure 1).

Vitamin D-dependent rickets (VDDR) is genetically heterogeneous depending on the disorders in vitamin D metabolism or action (60). VDDR type 1A (OMIM 264700) is due to an enzymatic defect in synthesis of the active 1,25(OH)₂D caused by mutations in the *CYP27B1* gene. VDDR1B (OMIM 600081) is a form of rickets due to mutations in the gene encoding the vitamin D 25-hydroxylase (*CYP27A1*), another enzyme necessary for the synthesis of active vitamin D₃. Hereditary vitamin D resistant rickets (HVDRR), also known as vitamin D-dependent rickets type 2, is an autosomal recessive disorder that is caused by end-organ unresponsiveness of active vitamin D, and in one case (VDDR2A) due to mutations in the VDR (OMIM 277440) (61-62). Alternatively, VDDR2B (OMIM 600785) is an unusual form of HVDRR whereby end-organ unresponsiveness to active vitamin D is due to dysregulation of hnRNP C1/C2 (i.e. the VDRE-BP) which interferes in a dominant negative manner with the function of VDR-DNA interactions (11-12,63). In both cases, the disorders are characterized by early onset severe rickets, hypocalcemia, secondary hyperparathyroidism, occasional alopecia, elevated 1,25(OH)₂D levels, and resistance to treatment with 1,25(OH)₂D (63-64).

hnRNPs are a family of ubiquitously expressed nuclear DNA- and (hn)RNA-binding proteins that share a wide array of cellular functions (65-67). At least 20 of these proteins, designated A through U, are present in abundance in the nucleus and are found in association with proteins to form hnRNP particles. The hnRNP family members and large number of accompanying isoforms serve to mediate pre-mRNA processing and mRNA splicing, packaging, export and stability as well as chromatin remodeling through locus control region-associated remodeling complexes, recombination, and telomere lengthening (65). The functional roles of hnRNP proteins influence the clinical and biological outcomes of viral infections (68-69), spinal muscular atrophy (70), myotonic dystrophy (71), Alzheimer's disease (72), tumorigenesis and hormone resistance (11-12,14,65).

In vertebrates, hnRNPC belongs to a subfamily of hnRNPs as a major constituent of 40S hnRNP particles; multiple transcript variants encoding at least two different isoforms (C1 and C2) have been described (<http://ensembl.org>). The hnRNPC protein acts as a tetramer [(C1)₃C2], and the two isoforms are antigenically closely related phosphoproteins which bind tightly to RNA *in vitro* (73-74). The hnRNPC proteins are divided into distinct domains: 1) a N-terminal highly conserved RNA-binding domain (RBD), also called RNA-recognition motif (RRM), 2) a central variable domain, and 3) a C-terminal auxiliary domain (67). It is possible that these individual regions on their own may have a unique structure and function. It is also possible that these regions may function cooperatively through interaction or physical linkage between individual regions.

HnRNP C1/C2 is known to play pleiotropic roles in regulating a number of biological activities ranging from the classic functions described above to the direct inhibition of

apoptosis by interacting with multiple proteins (75), although much of the mechanism remain unknown. Despite their classical interaction with chromatin-associated single-stranded RNA, several studies have shown that hnRNPs are pluripotent proteins. In particular, they may also function as transregulatory factors through interaction with double-stranded DNA (33), raising the question as to whether they may also be part of the normal VDR-mediated gene regulation machinery. ChIP studies from normal 1,25(OH)₂D-sensitive cells indicated that this is indeed the case, with the human hnRNP C1/C2 occupying the VDRE of the major vitamin D-target gene *CYP24A1* (1,25-dihydroxyvitamin D₃ 24-hydroxylase), involved in metabolite catabolism, prior to treatment with 1,25(OH)₂D, and then being displaced from the promoter by the liganded VDR-RXR complex (11). This reciprocal relationship between VDR and VDRE-BP occurs in a time-dependent cyclical fashion following exposure to 1,25(OH)₂D, suggesting that hnRNP C1/C2 plays a pivotal role in the spatio-temporal organization of the receptor-response element complex. Importantly, hnRNP C1/C2 was shown to regulate 1,25(OH)₂D actions in a patient with a form of HVDRR due to its cellular over expression resulting in a phenotype similar to UVB-restricted NWP cells investigated previously (11-12,63). In this experiment of nature, over abundance of hnRNP C1/C2 was shown to interfere with the VDR binding to the VDRE located in the proximal promoter region of *CYP24A1*. Subsequently through *in vitro* over expression studies using a transcriptional reporter system, hnRNP C1/C2 was shown to inhibit vitamin D transactivation, providing further evidence of such mechanism. Chen et al. (12) proposed that vitamin D resistance in this patient was similar to that described in NWP cells, in which abnormal expression of a HRE-binding protein caused target cell resistance to 1,25(OH)₂D (76).

VII. Estrogen response element binding protein (ERE-BP) and estrogen resistance

NWP cells exhibit resistance to other steroid hormones in addition to vitamin D, notably exhibiting squelching of normal estradiol-ER-ERE-directed transcription (9). As a result, studies similar to those employed for analysis of vitamin D resistance in NWP cells have been performed to assess nuclear ER signaling. In this case, concentrated nuclear extracts from estrogen resistant NWP cells were screened and the hnRNP C-like protein was identified as the ERE-BP (15). The functionality of the ERE-BP was demonstrated in studies both *in vitro* and *in vivo* (13,51). In the case of the latter, transgenic mouse lines with varying degrees of ERE-BP over-expression were generated and confirmed that tissue-specific over-expression of ERE-BP results in estrogen resistance *in vivo* (51). Given the fundamental role of estrogen in reproduction, it was reasoned that global over-expression of an estrogen-resistance gene was likely to have lethal consequences. All offspring born to wild-type and 'low' ERE-BP-expressing mothers were viable, whereas one third of all pups born to mothers with 'intermediate' ERE-BP died before weaning. Significantly, 100% of offspring born to 'high' ERE-BP mothers died by postnatal day 7 (51). Further analysis showed that mortality was due to starvation as a result of decreased ingestion of breast milk ERE-BP-overexpressing mothers; a rebound increase in body weight was observed in pups born of high ERE-BP-expressing mothers if fostered to a wild-type mother.

Histological analysis of mammary glands from either virgin or lactating female mice showed that ERE-BP over-expression resulted in decreased numbers of mammary gland ducts and branches. It was proposed that increased ERE-BP expression led to E₂ insensitivity and impaired breast development. Parallel studies performed *in vitro* indicated that over-expression of ERE-BP dramatically blunted responses to E₂, although it was possible to induce significant ERα-ERE signaling in ERE-BP over-expressing cells if one used E₂ in high amounts to drive breast development and lactation (51).

Based on the above described observation that E₂ could rescue the ERE-BP-mediated suppression of ER α -driven gene expression *in vitro*, further studies using ERE-BP transgenic mice were performed in which animals were provided with slow-releasing subcutaneous E₂ pellets to differentially elevate serum E₂ levels (76). Whole mount and histological analyses of breast tissue showed that exposure to higher supplemental doses of E₂ “rescued” the impaired mammary gland development in ERE-BP over-expressing mice. Rescue of mammary gland development in virgin non-lactating female mice as well as in lactating mothers was accomplished with E₂, indicating that the dominant-negative effect of ERE-BP was active at different stages of breast function. Significantly and unexpectedly, these studies showed that tamoxifen was as effective as E₂ in counteracting the inhibitory effects of ERE-BP, suggesting that the selective estrogen receptor modulator (SERM) is capable of exerting effects on estrogen action that are distinct from its effects on ER α or its accessory, co-regulatory proteins. Further clarification of this observation was obtained in experiments done *in vitro* in which the ability of ER α and ERE-BP to interact with chromatin was assessed by ChIP analysis of wild-type and ERE-BP-over-expressing MCF-7 breast cells treated with E₂ or tamoxifen (76). As with the hnRNP C1/C2 VDRE-BP (11), cells over-expressing ERE-BP showed dysregulation of the spatio-temporal patterns of ERE occupancy; both E₂ and tamoxifen were able to correct this dysregulation and promote binding of ER α to a pS2 gene ERE. These findings confirmed the reversible nature of the physical interaction between hnRNP C-like proteins and receptor *cis* regulatory elements.

VIII. Conclusion

With time, the mechanisms underlying the control of hormone action and metabolism within target cells continue to expand, sometimes in unexpected directions. The discovery of the hnRNPC-related vitamin D and estrogen response element binding proteins in subhuman primates and in humans is such a development. The potential for these multifunctional nucleic acid binding proteins to alter hormone-directed transcription of genes in addition to controlling the post-transcriptional fate of mRNAs transcribed from those genes indicates that the traditional views of the various nuclear machines (i.e. transcriptosome, spliceosome, etc) working independently of one another may need revision. Future studies will be aimed at clarifying the mechanisms by which VDRE-BP and ERE-BP bind to putative gene promoter HREs to direct gene transcription for critical VDR and ER target genes. Similar mechanisms also appear to be central to signaling by other steroid hormones, providing further support for these novel co-facilitators and co-modulators as key components of nuclear receptor signaling.

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X. Abbreviations

NWP	New World Primate
OWP	Old World Primate
VDRE-BP	Vitamin D response element binding protein
ERE-BP	Estrogen response element binding protein
hnRNP	heterogeneous nuclear ribonucleoprotein
HVDRR	hereditary vitamin D resistant rickets

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Figure 1. Steroid receptor induction of gene transcription: a multi-step control model of VDR activity at the genomic and post-translational levels

1 α ,25-dihydroxyvitamin D₃ (vitamin D₃) is a hormone that plays a crucial role in the regulation/metabolism of calcium and phosphorus in the small intestine, kidney and bone. In addition it is also involved in immune function, tumor suppression, growth regulation and parathyroid hormone secretion. Serving an endocrine role, free (i.e. unbound) steroids can enter the cell cytoplasm and interact with their receptor. For certain cell types, vitamin D₃ can be self synthesized in the cytoplasm. In this process heat shock proteins are dissociated, and the activated receptor-ligand complex is translocated into the nucleus. VDR, the vitamin D₃ receptor, exists as a heterodimer with RXR. Vitamin D₃-bound VDR-RXR, along with other co-activator and co-repressor proteins, mediates the transcriptional regulation of a number of genes in the nucleus. Importantly, VDRE-BPs also mediate transcription of VDR-responsive genes involved in hormone, bone and growth regulation. Hence any disruption to the vitamin or its receptor would have consequences in a number of the key physiological processes. Furthermore at the post-receptor level, defective co-activator or excessive co-repressor activity could lead to nuclear hormone resistance affecting multiple nuclear hormonal receptor systems. Data were analyzed and validated (e.g. citations) through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com)

Table 1

Vitamin D and estrogen steroid receptor superfamily

Subfamily	Group	Name	Ligand	Function	OMIM ¹	Disorder/Resistance ²	Selected References
Thyroid hormone receptor-like	Vitamin D receptor-like	Vitamin D receptor (VDR) (VDR)	-Vitamin D (1,25(OH) ₂ D) -Lithocholic acid	-Mineral transport and metabolism -Immune response -Cancer progression -Decidualization -Intestinal absorption -Skeletal development -Hair development	601769	-Hereditary vitamin D-dependent rickets type 2A -Hyperparathyroidism secondary -Osteoporosis -Vitamin D deficiencies -Prostate/breast cancer -Alopecia -Acute lymphocytic Leukemia	(53,86-95)
Estrogen receptor-like	Estrogen receptor	Estrogen receptor- α, β (ER α, β) (ESR1,2)	- Estrogens	-Estrous cycle -Sexual development -Reduce muscle mass -Increase fat stores -Reduce bone resorption, increase bone formation -Coagulation -Lipid metabolism -Cancer -Lung function	133430 601663	-Estrogen resistance -Breast cancer -Endometrial cancer -Uterine leiomyomas -Familial hypercholesterolemia	(96-101)

¹ OMIM® (Online Mendelian Inheritance in Man) - catalog of mendelian traits and disorders

² Some on the links under disorder/resistance are suggestive, as with vitamin D status for the risk of prostate and breast cancers

Hormone response element interactors

Table 2

Associating Nuclear Receptor	Binding Protein/Compound/ncRNA	Response Element	Regulated Gene(s)	Binding Factor Effects	Reference(s)
ROR α,β,γ	Rev-ErbA beta (NR1D2)	ROR α RE	HRE-reporter gene	competitive repressor of ROR α function	(36)
LXR α,β	thyroid hormone receptor- β 1	LXRE	sterol regulatory element binding protein 1c	suppression of LXR α transactivation	(37)
FXR	steroidogenic factor 1	AGGTCA	aromatase	basal factor that is competed by FXR which acts negatively on the promoter	(39)
VDR	YY1		osteocalcin	repression of VDR-RXR-TFIIIB-mediated transactivation	(29)
	hnRNP C1/2	VDRE	CYP24A1, DDIT4, osteocalcin ¹	repression of VDR-RXR mediated transactivation	(10-12,102)
ER α,β	scaffold attachment factor B	ERE-TATA	heat shock protein 27	over expression leads transcriptional suppression	(30)
	hnRNP C-like 1	ERE	cathepsin D	suppression of ER-ERE-mediated transactivation	(13-14)
GR	growth arrest-specific transcript 5 (riborepressor)	decoy GRE	inhibitor of apoptosis 2		(40)
	polyamide	GRE	GC-induced leucine zipper (GILZ) gene	GR activity is repressed	(41)
	AP-1	plfG ¹	proliferin	enhancement or repression depending on AP-1 composition	(32)
AR	polyamide	ARE	prostate-specific antigen	suppression of androgen receptor-mediated gene expression	(42)

¹ Composite hormone response element consisting of GR and alternating AP-1 binding sites