Novel approaches for the study of vertebrate steroid hormone receptors

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Synopsis Steroid hormones are essential for the normal function of most organ systems in vertebrates. Reproductive activities in females and males, such as the differentiation, growth and maintenance of the reproductive system, require signaling by sex steroid hormones. Although extensively studied in mammals and a few fish and bird species, the evolution and molecular mechanisms associated with the nuclear steroid hormone receptors are still poorly understood in amphibians and reptiles. Given our interest in environmental signaling of sex determination as well as a major interest in environmental contaminants that can mimic steroid hormone signaling, we have established an approach to study the molecular function (ligand binding and trans-activation) of steroid hormone receptors cloned from reptiles. This approach involves molecular cloning and sequencing of steroid hormone receptors, phylogenetic analysis and in vitro trans-activation assays using endogenous or exogenous ligands. Comparing the in vitro trans-activation induced by different ligands with receptors cloned from different species would develop additional functional relationships (classification) among steroid hormone receptors. This approach can provide insight into understanding why each species could have different responses to exogenous ligands. Further, we have developed a novel and less invasive approach to obtaining mRNA for molecular cloning and sequencing of steroid hormone receptors in reptiles and other non-mammalian species, using blood cells as a source of genetic material. For example, white blood cells (WBCs) and red blood cells (RBCs) of the American alligator both express steroid hormone receptors and have adequate amounts of mRNA for molecular cloning. This approach would allow us to analyze components of endocrine function of steroid hormones without sacrificing animals. Especially in endangered species, this approach could provide an understanding of endocrine functions, elucidate the phylogenetic relationships of various receptors in vitro, such as the steroid hormone receptors, and determine possible effects of environmental contaminants in a minimally invasive manner.

Introduction

Steroid hormones are essential for the normal function of most organ systems in vertebrates. Estrogens regulate ovarian development and differentiation, induce maturation of the female reproductive tract, and stimulate hepatic vitellogenesis in reptiles. Androgens are involved in testicular development and differentiation, control of spermatogenesis, maturation of the male reproductive tract and differentiation of external genitalia (Norris 1980; Bentley 1998).

The differentiation, growth, and maintenance of the reproductive system require endocrine signaling by sex steroid hormones via their receptors. Two different types of receptors for steroid hormones have been described: those that belong to the super family of nuclear receptors (Blumberg and Evans 1998; Bertrand et al. 2004) and those that function as membrane-linked (extra-nuclear) receptors (Hammes and Levin 2007). Two subtypes of receptor proteins have been described to represent the extra-nuclear version: G-coupled steroid hormone receptors (Filardo et al. 2000; Zhu et al. 2003; Revankar et al. 2005; Thomas et al. 2006) and membrane-localized classical steroid hormone receptors (Razandi et al. 2003; Pedram et al. 2007). Nuclear receptors work as transcription factors regulating mRNA expression, and have six functionally distinct domains (Krust et al. 1986). These domains are labeled the A through F domains with the C and E domains playing essential roles of DNA binding and ligand binding, respectively (Fig. 1). The C domain of a steroid receptor is the DNA-binding domain, which uses a zinc finger motif to accomplish binding to target DNA. The E domain
is the ligand-binding domain, which is involved in binding to steroid hormone and recruiting various transcriptional cofactors to the steroid receptor so that transcription can occur (Pratt and Toft 1997). Extra-nuclear receptors for steroid hormones have been demonstrated and various receptor types have been cloned. The rapid reactions to steroid hormones characterizing these membrane receptors, as well as membrane-localized classical steroid hormone receptors, appear to be induced by G-protein coupling, which causes post-translational modifications or phosphorylation (Razandi et al. 2003; Hammes and Levin 2007; Watson et al. 2007).

Although, extensively studied in mammals and in a few fish and bird species, the evolution and molecular mechanisms associated with the nuclear steroid hormone receptors have been poorly understood, especially in amphibians and reptiles (Young et al. 1995). For example, it is well accepted that the estrogenic sex steroid hormones play an important role in sex determination in many non-mammalian and non-avian vertebrates (Crews 2003; Yao and Capel 2005).

In mammals and birds, sex determination is dependent on the genotype, XX:XY or ZZ:ZZ, at fertilization. However, in some lizards, and turtles and all crocodilians studied to date, sex determination is dependent on incubation temperature during a narrow time period of embryonic development (Crews 2003). Generally, lower temperatures produce males whereas higher temperatures produce females in turtles. In contrast, lower and very high temperatures produce females and intermediate temperatures produce males in crocodilians (Crews 2003). Sex determination by environmental signals has been investigated, and the important role of sex steroid hormones on sex reversal in reptilian sex determination has been established (Crews 2003).

Given this sensitivity of sex determination to steroid hormone signaling, contamination of the global environment with chemicals that can mimic steroid hormones should be a major concern. It has been well established that some environmental contaminants can act as agonist or antagonist ligands on various steroid hormone receptors or they can alter the synthesis/degradation of endogenous hormones (Tyler et al. 1998; Guillette and Gunderson 2001; Milnes et al. 2006). Several studies suggest that various contaminants can directly act on steroid hormone receptors as an agonist or antagonist (Rooney and Guillette 2000; Gray et al. 2002). Indeed, endocrine alterations which result from developmental exposure to endocrine disruptive contaminants has been observed in a number of wildlife species, including turtles (Willingham et al. 2000) and the American alligator (Guillette et al. 2000; Guillette et al. 2007). Exposure to various contaminants with estrogenic potential during development alters sex determination and gonadal steroidogenesis in freshwater turtles (Willingham and Crews 1999; Willingham et al. 2000) and the American alligator (Crain et al. 1997). Further, recent studies have documented altered expression of estrogen receptor β (ESR2) mRNA in gonadal tissue from juvenile American alligators caught in Lake Apopka (FL, USA) (Kohno et al. 2008). This lake is polluted with various endocrine disruptive contaminants, some of which have been shown to be estrogenic (Vonier et al. 1996; Guillette et al. 2002). Male gonadal tissue from animals obtained at Lake Apopka exhibited elevated expression of ESR2 mRNA when compared to animals of similar age and size from a reference population (Kohno et al. 2008). The altered expression and functioning of ESR2 might be important in explaining the gonadal alterations induced by contaminants in the American alligator as previous studies have suggested that this receptor type is reactive with a
A wide array of exogeneous estrogenic chemicals (Kuiper et al. 1996). Therefore, functional details of steroid hormone receptors in wildlife, especially in reptiles, need to be investigated. Here, we summarize several novel approaches to studying the molecular function (ligand binding and trans-activation) of steroid hormone receptors cloned from reptiles and fishes.

**Two different trans-activation assays for examining steroid receptors in vitro**

Reporter gene assays using luciferase in mammalian cell lines are commonly used to characterize trans-activation by steroid hormone receptors. Generally, the cells are transfected with plasmids including cDNA for a steroid hormone receptor. The reporter plasmids, which have a specific sequence of a hormone response element (HRE) and luciferase reporter cDNA, are also transfected into the cells (Fig. 2). Following ligand (steroid hormone or test chemical) binding to the receptor, the receptor–ligand complex interacts with the HRE, inducing the transcription of luciferase cDNA. As a result, the reporter protein, luciferase, accumulates in the cells and the luminescence signal increases in a dose-dependent manner (Fig. 2). However, these results would be somewhat limited as they indicate potential trans-activation using only a canonical HRE. Generally, investigators use a common sequence of HRE for analysis of steroid hormone receptors cloned from any vertebrate, although it’s rare to find canonical HREs in the promoter region (O’Lone et al. 2004). The canonical HREs do not produce problems in the trans-activation assay; however, each steroid hormone receptor from different species could have differing responses to the canonical HRE given the variation in amino-acid sequence and folding of each unique receptor. Therefore, we have added to this approach a new assay, the modified GAL4 system that does not require DNA recognition of an HRE. To eliminate this dependence on the canonical sequence of the HRE, we have developed a novel reporter–gene assay using a modified GAL4 system (Promega, Madison, WI) that is traditionally used in the two-hybrid system for mammalian cells (Katsu et al. 2006a). The cells are transfected with the expression construct for a steroid hormone receptor-GAL4 fusion protein. Following ligand binding to the receptor, and if it can induce trans-activation, the receptor-GAL4 fusion protein interacts with the GAL4 binding site and activates transcription. As a result, luciferase accumulates in the cell and a luciferase signal increases with the dose of the ligand (Fig 2). The modified GAL4 system, using red-belly turtle (Pseudemys nelsoni) ESR1, for example, exhibits a 10-fold higher induction with estradiol-17β when compared to the HRE-Luciferase System; however, the sensitivity between the two assay
systems, measured as an EC50 for example, is not different (Katsu et al. 2008). The two assay systems use different mammalian cell lines, as HEK 293 or Hep G2 were used for the trans-activation assay with HRE or AR, whereas CHO-K1 cells were used for the GAL4 trans-activation assay. This difference in the fold induction of luciferase induced by the various tested ligands could be caused by different regulatory sequences of the luciferase reporter cDNA (HRE versus GAL4) or by differing cell lines (HEK293 or Hep G2 cells for HRE; CHO-K1 for GAL4). Moreover, this new system, as described above, is completely free of HRE sequence issues. However, one potential issue with this system relates to the effect of fusing the steroid hormone receptor and GAL4 protein on trans-activation. Although both systems need to recruit cofactors for trans-activation, recruiting the cofactors could be a potential problem with the steroid hormone receptor-GAL4 fusion protein. The transfected plasmids of the luciferase reporter cDNA have only a limited regulatory region such as the HRE or the GAL4 binding site to express luciferase and don’t have AP1 or Sp1 sites. Therefore, it is possible to miss potential inductions of trans-activation via action of membrane-localized, classical, steroid-hormone receptors, or other transcription factors working with steroid–hormone receptors, such as Sp1 or AP1.

Estrogen receptor α (ER α; ESR1)

Phylogenetic analysis of ESR1 amino-acid sequences clearly has shown that crocodilian estrogen receptors are a sister group of Aves and form an archosaurian clad (Katsu et al. 2004; Katsu et al. 2006b). Red-belly turtle (P. nelsoni) is in an immediate out-group of Archosauromelia with the most distinct group of squamates (lizards and snakes) when birds and reptiles are compared (Katsu et al. 2008). Ten ESR1 sequences have been cloned from Sauropsida (birds and reptiles); however, to date, only four ESR1 sequences have been characterized by trans-activation. The DNA-binding and ligand-binding domains of ESR1 are well conserved among vertebrate species compared with amino-acid sequences (Fig. 1A). Specifically, the DNA-binding domain is highly conserved with 94–100% identity among vertebrates. This conservation of sequence suggests that ESR1 could regulate similar target genes among vertebrate species. However, this needs to be tested and further clarification is possible, once trans-activation data are available, from assays of ESR1 action as described above. Similarly, the ligand-binding domain is well conserved, but it does not show the same very high degree of sequence similarity as seen in the DNA-binding domain (Fig. 1A). This indicates that ESR1 could potentially bind to a wide range of ligands with species specificity.

Trans-activation assays using red-belly turtle (P. nelsoni) and roach (Rutilus rutilus) ESR1 revealed that estradiol-17β (E2), ethynylestradiol (EE2), and diethylstilbestrol (DES) have higher potentials than estrone (E1) or estriol (E3), whereas DES showed higher potentials than E2 in mosquitofish (Gambusia affinis affinis) (Fig. 3). As a unique way of examining steroid hormone receptor activity, we performed a cluster analysis using each EC50 in the trans-activation assay of ESR1 (Fig. 5A). This analysis showed that mosquitofish ESR1 has more similar characteristics to red-belly turtle ESR1 than to roach ESR1, as ligands were classified into two groups by their characteristics of trans-activation; that is, group of E2, EE2, and DES and group of E1 and E2 (Fig. 5A). This initial analysis is small as it used just three species and five ligands, but is promising in that it could provide important

Fig. 3 The trans-activation assay on (A) red-belly turtle, (B) mosquitofish, and (C) roach estrogen receptor α (ESR1). Red-belly and roach ESR1 revealed that E2, EE2, and DES have higher potentials than does E1 or E3, whereas DES showed higher potentials than did E2 in mosquitofish ESR1. Charts were modified from publications by Katsu et al. (2007a, 2007b, 2008).
predictive information about species risk to various environmental contaminants.

**Androgen receptor (AR)**

A recent phylogenetic analysis of AR amino-acid sequences in reptiles and birds has been presented (Katsu et al. 2008). Phylogenetic relationships for the ARs, based on molecular sequences, were similar to those observed for ESR1. There are only six sequences of full-length AR cloned from Sauropsida in the database, and only three receptors have been characterized by trans-activation. The DNA-binding and ligand-binding domains of AR are well conserved among vertebrates (Fig. 1B). Trans-activation of the red-belly turtle AR with a variety of endogenous (DHT, 5α-dihydrotestosterone; T, testosterone; 11-KT, 11-ketotestosterone) and pharmaceutical androgens (MT, 17α-methyltestosterone; Tren, trenbolone) revealed the following order in trans-activation potential: DHT = MT = Tren > T (Figs. 4 and 5B). Mosquitofish (G. affinis affinis), like other teleost fish studied to date, have two ARs: type-1 and type-2 (Katsu et al. 2007a). Trans-activation on both types of mosquitofish AR revealed a similar pattern; that is, MT had the highest potential, whereas Tren = 11-KT > DHT > T (Figs. 4 and 5B). However, cluster analysis of ARs using their EC50 for different ligands revealed a different relationship than expressed by the phylogeny based on amino-acid sequences (Fig. 5B). On the other hand, DHT and T showed different characteristic from MT, Tren and 11-KT on trans-activation of AR in the cluster analysis of ligands (Fig. 5B).

Although more data, both from additional species and ligands, are needed to obtain better estimates of classifications, these results indicate that the functional characteristics of recombinant protein could be a great source of data to estimate novel physiological responses that depend on the functions of cloned proteins. Considering both sequence-based and functional-characteristics-based clustering or classification could provide help in understanding not only endogenous endocrine systems, but also be used to develop risk assessments for vertebrate species exposed to a wide array of endocrine-altering environmental contaminants.

**A novel approach for cDNA cloning and analysis of steroid receptors**

Most non-mammalian species, including alligators, have RBCs with a functional nucleus and organelles similar to other cells (Kregenow 1977); thus, RBCs likely regulate mRNA expression as do other type of cells. Therefore, both WBCs and RBCs are likely to respond to endogenous/exogenous environmental factors at the mRNA level. They could be a potential source of data on environmental perturbation and have enough mRNA to analyze by current molecular biological techniques such as cDNA cloning or quantitative real-time PCR. Collection of blood samples and analysis of plasma are very common techniques, even for endangered species. If the investigators saved the blood cells properly after they transfer the plasma in any non-mammalian species, including endangered species, a component of the endogenous endocrine system could be studied. This could open various directions in research, such as comparing mRNA expression in blood cells with hormone concentrations in the plasma or comparing alterations in mRNA expression over bleeds. Indeed, in the American alligator, stress from constraint increased the concentrations of corticosterone and glucose.

Fig. 4 The trans-activation assay on (A) red-belly turtle, (B) mosquitofish type-1, and (C) type-2 androgen receptor (AR). Charts were modified from publications by Katsu et al. (2007a, 2007b, 2008).
in plasma. Glucocorticoid receptor (GR) mRNA in whole blood cells collected from the same blood was detectable by quantitative real-time PCR, and GR mRNA was slightly induced at 8 h after stress from restraint (Kohno and Guillette, unpublished data). Further, the blood cells expressed enough mRNA to clone many of the steroid hormone receptors (e.g., ESR1, GR or AR). These results indicate that it would be possible to obtain and analyze the trans-activation of steroid hormone receptors cloned from blood cells of endangered species and further allow determination of whether a given species is at greater risk to environmental contaminants with agonistic or antagonistic steroid hormone potential. Such a study is in its initial stages for the Japanese Giant Salamander (Andrias japonicus), a national monument of Japan and a highly endangered species (Katsu et al. 2006a).

**Conclusion**

Two novel applications for cDNA cloning and trans-activation assays could make the functional analysis of steroid hormone receptors from endangered species possible. Cluster analysis, using ligand-receptor trans-activities is likely to provide new functional aspects of receptor-ligand interactions by grouping similar receptor function and/or ligand potential. Furthermore, it might be possible to estimate effects of, and risk to environmental contaminants via nuclear steroid hormone receptors on many vertebrate species, including threatened or endangered species.

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