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Comparative Biochemistry and Physiology, Part C 148 (2008) 381–389

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Review

Candidates for membrane progestin receptors—Past approaches and future challenges ☆

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ARTICLE INFO

Article history:
Received 9 March 2008
Received in revised form 19 May 2008
Accepted 20 May 2008
Available online 15 June 2008

Keywords:
Nongenomic
Membrane steroid receptor
Membrane progestin receptor
mPR
Nuclear progestin receptor
Progestin receptor membrane component
PGRMC
Ovary
Oocyte
Zebrafish

ABSTRACT

Progestins have a broad range of functions in reproductive biology. Many rapid nongenomic actions of progestins have been identified, including induction of oocyte maturation, modulation of reproductive signaling in the brain, rapid activation of breast cancer cell signaling, induction of the acrosomal reaction and hypermotility in mammalian sperm. Currently, there are three receptor candidates for mediating rapid progestin actions: (1) membrane progestin receptors (mPRs); (2) progestin receptor membrane components (PGRMCs); and (3) nuclear progestin receptors (nPRs). The recently-described mPR family of proteins has seven integral transmembrane domains and mediates signaling via G-protein coupled pathways. The PGRMCs have a single transmembrane with putative Src homology domains for potential activation of second messengers. The classical nPRs, in addition to having well defined transcriptional activity, can also mediate rapid activation of intracellular signaling pathways. However, details of the mechanisms by which these three classes of progestin receptors mediate rapid intracellular signaling and their subcellular localization remain unclear. In addition, mPRs, nPRs and PGRMCs exhibit overlapping expression and functions in multiple tissues, implying potential interactions during oocyte maturation, parturition, and breast cancer signaling in individual cells. However, the overwhelming majority of studies to date have focused on the functions of one of these groups of receptors in isolation. This review will summarize recent findings on the three major progestin receptor candidates, emphasizing the different approaches used, some experimental pitfalls, and current controversies. We will also review evidence for the involvement of mPRs and nPRs in one of the most well-characterized nongenomic steroid actions in basal vertebrates, oocyte maturation, and conclude by suggesting some future areas of research. Clarification of the controversies surrounding the identities and localization of membrane progestin receptors may help direct future research that could advance our understanding of rapid actions of steroids.

Contents

☆ Contribution to the Special Issue of CBP on Chinese Comparative Biochemistry and Physiology presented at or related to the International Conference of Comparative Physiology, Biochemistry and Toxicology and the 6th Chinese Comparative Physiology Conference, October, 10–14, 2007, Zhejiang University, Hangzhou, China.

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doi:10.1016/j.cbpc.2008.05.019

1. Introduction

Nuclear steroid receptors (nSR) are primarily localized in the nucleus or cytoplasm of cells. These intracellular receptors are activated by the binding of steroids, which are hydrophobic and freely diffuse through the cell membrane. The ligand-receptor complex is translocated to the nucleus, where it binds to hormone response elements on genes, resulting in changes in their rates of transcription and translation. This classical genomic mechanism of steroid action, involving the transcription of DNA and synthesis of proteins, is a relatively slow process that can take hours to days to elicit a biological response. However, unlike the classical steroid mechanism, many actions of steroids occur much more rapidly (<30 min) and in the presence of inhibitors of transcription and translation. These rapid, nongenomic or nonclassical steroid actions have been well recognized and extensively studied over the past few decades (Pietras and Szego, 1975; Norman et al., 2004; Thomas 2008).

Nonclassical rapid steroid actions have been demonstrated for all the major classes of steroids (Norman et al., 2004). Well known examples of nonclassical progestin actions include induction of the acrosomal reaction in sperm (Blackmore et al., 1990; Sabeur et al., 1996; Baldi et al., 1998; Luconi et al., 2004), rapid increases in sperm motility (Thomas et al., 2005), modulation of gonadotropin-releasing hormone discharge in the brain (Majewska et al., 1986; Calogero et al., 1998; Sim et al., 2001), rapid activation of breast cancer cell signaling (Faivre et al., 2005), and resumption of oocyte maturation in fish and amphibian species (Kostellow et al., 1980; Ferrell 1999; Thomas et al., 2002). Interestingly, some of these actions occur in the absence of the nuclear progesterone receptor (nPR). For example, progestins activate signal transduction pathways in human T lymphocytes and Jurkat cells that lack nPR (Dosiou et al., 2008). In addition, rapid behavioral responses to progesterone such as lordosis persist in nPR-null mice (Frye et al., 2006). Rapid, nongenomic actions of progestins on gametes have been investigated extensively (Maller 2001; Thomas et al., 2004). Furthermore, specific steroid binding sites have been identified on plasma membranes prepared from oocytes and sperm implying membrane localization and activity of progestin receptors (Patiño and Thomas 1990; Blackmore and Lattanzio 1991). Although these studies have provided strong evidence for the existence of specific membrane receptors, the identities of progestin receptors mediating these effects remain controversial (Lösel et al., 2003; Norman et al., 2004; Thomas 2008).

In this paper we will review the following major candidates for progestin receptors mediating the nongenomic actions of progestins, with an emphasis on their effects on gametes: (1) membrane progestin receptors (mPRs) (Zhu et al., 2003a,b); (2) progesterone receptor membrane component 1 (PGRMC1) (Falkenstein et al., 1996; Meyer et al., 1996); and (3) nuclear progestin receptors (nPRs) (Bayaa et al., 2000; Tian et al., 2000). For information on steroid receptor candidates mediating nongenomic actions of other steroids, please refer to other recent reviews (Hammes and Levin 2007; Stormshak and Bishop, 2008). Approaches used in the past to identify and characterize membrane progestin receptors, and the controversies surrounding their identities, localization and signaling will be reviewed. In addition, evidence in support of mPRs and nPRs as the receptors mediating the effects of maturation-inducing steroids on oocyte maturation in basal vertebrates is discussed.

2. Oocyte maturation—a model for nongenomic progestin actions

Prior to oocyte maturation, which is a prerequisite for successful ovulation and fertilization, full-grown immature oocytes are arrested indefinitely at prophase I of meiosis (Yamashita et al., 2000; Maller 2001; Thomas et al., 2002). During oocyte maturation, meiosis resumes, the germinal vesicle breaks down, chromosome condensation occurs, and the first polar body is assembled. It has been known for several decades that specific steroid hormones called maturation-inducing steroids (MIS) are capable of triggering the resumption of meiosis and final oocyte maturation in amphibian and fish species. The MIS is released from the granulosa layer surrounding the fully-grown immature oocytes in response to stimulation by luteinizing hormone. Over 40 years ago, Masui (1967) identified progesterone as an inducer of meiosis in amphibian oocytes. Subsequently, two different specific progestins identified in fish species were shown to act at the oocyte membrane to induce oocyte maturation more effectively than progesterone. In perciform fish such as the spotted seatrout, 4-pregnen-17,20β,21-triol-3-one (20β-S) was the most potent MIS (Trant et al., 1986; Thomas and Trant 1989), while 17α,20β diol-3-one (17α,20β) serves as the MIS in salmon (Nagahama and Adachi 1985) and zebrafish (van den Hurk et al., 1987).

3. Obstacles in identifying membrane progestin receptors

Actions of the MIS are very rapid (less than 1 min) (Thomas and Das, 1997) and are mediated via specific progestin receptors localized on the oocyte membrane (Patiño and Thomas, 1990). However, technical obstacles in isolating functional membrane progestin receptors, and also possibly the adoption of somewhat limited, focused approaches, have thwarted attempts to identify the full suite of progestin receptors that could be potentially involved in a particular nonclassical progestin action. Some investigators have attempted to identify progestin receptors that are intermediaries on nonclassical progestin actions using PCR primers, antibodies, and cDNAs based on the sequence and characteristics of nPRs. In general, their results support the conclusion that the classical nPRs are the receptors responsible for nongenomic actions of progestins in a variety of cells types, including *Xenopus* oocytes and human breast cancer cells (Bayaa et al., 2000; Tian et al., 2000; Faivre et al., 2005). In contrast, other researchers, on the basis of their results showing that the biochemical binding characteristics of the receptors in their models differ from those of nPRs, have attempted to identify novel proteins, unrelated to nPRs, with the characteristics of membrane progestin receptors (Falkenstein et al., 1996; Zhu et al., 2003b). Evidence for the presence of novel membrane progestin receptors is supported by the persistence of nongenomic actions in nPR-null mice and cell lines (Lydon et al., 1996; Frye et al., 2006; Dosiou et al., 2008). Nonetheless, attempts to identify and biochemically purify specific membrane steroid receptors mediating nongenomic effects have been greatly impeded due to a number of reasons including the intrinsic properties of the receptors themselves and technical difficulties in their purification. Of course, appropriate probes for monitoring the activity and identity of these progestin receptors during the purification process were lacking initially due to the absence of information on their identities. The identification of the progestin receptor-enriched fractions during purification by radioreceptor assay is complicated by the high non-specific binding of progestins to membrane fractions due to their lipophilic nature. Non-specific binding of progestins can range from several-hundred to several-thousand dpm depending on the assay conditions. Moreover, there is also high non-specific binding of steroids to detergents commonly used for extraction of the membrane steroid receptors (Fig. 1). The

presence of high non-specific progesterone binding to membranes and detergents makes it difficult to evaluate the efficacy of different purification steps and may lead to false positives in assays measuring receptor binding. In addition, the amounts of some tissues such as human sperm and amphibian brain that can be readily obtained are limited, making large-scale purification of receptors from them tedious (Evans et al., 2000; Luconi et al., 2002). Another problem encountered during purification of mPRα from fish ovaries was the rapid loss of progestin binding activity during the purification process, presumably due to rapid degradation of the mPR following extraction of the receptor from the plasma membrane. Finally, monitoring binding activities of the purified fractions is technically challenging due to rapid association and dissociation of steroid binding to the receptors (Patiño and Thomas 1990; Zhu et al., 2003a,b). Careful consideration of all these obstacles is required in order to succeed in identification and/or characterization of membrane progestin receptors.

4. Isolation and purification of mPRα from spotted seatrout ovaries

Early studies on the hormonal control of oocyte maturation in spotted seatrout and a closely-relate sciaenid species, Atlantic croaker had positively identified a novel progestin, 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) as the maturation-inducing steroid (MIS) in these teleost species (Trant et al., 1986; Trant and Thomas 1988; Thomas and Trant 1989; Trant and Thomas 1989a,b). Subsequently, a membrane filtration assay protocol originally developed for biochemical characterization of a membrane glucocorticoid receptor in newt brains (Orchinik et al., 1992), was adapted for measurement of membrane progestin receptor activity in spotted seatrout ovarian membranes (Patiño and Thomas 1990). The rapid filtration assay system contains a holder for a large pore size glass-fiber filter that retains plasma membranes. The advantage of this assay is that the binding reaction can be terminated rapidly, and non-specific binding can be stripped off with large amounts of wash buffer that can be aspirated through the filter within seconds using a vacuum pump. These features of the rapid membrane filtration assay were extremely important for characterizing the ovarian membrane progestin receptor due to its rapid rates of ligand association / dissociation and the high levels of non-specific steroid binding associated with the plasma membranes. The first comprehensive characterization of the oocyte progestin membrane receptor in a teleost model was described in spotted seatrout using this membrane filtration assay (Patiño and Thomas 1990). These studies showed that the biochemical and steroid binding properties of the fish oocyte membrane progestin receptor were significantly different from nPR in this species (Patiño and Thomas 1990; Pinter and Thomas 1995). The rapid dissociation / association rates for the seatrout membrane progestin receptor (<10 min) versus slow dissociation/association rates for the seatrout nPR (1–1.5 h) is a key characteristic difference worth emphasizing here. These results suggesting that the membrane receptor may be a novel protein unrelated to the nuclear receptor prompted an attempt at its purification, even though this was extremely challenging due to a lack of specific tools such as antibodies. First a suitable assay for detecting solubilized steroid receptors during the purification process was developed. Optimization of the membrane filtration assay protocol using smaller pore sizes and higher protein retention filters (0.22 μ Millipore MF filter) improved the assay and reduced variability. In addition, a modification of an assay developed to characterize soluble steroid binding proteins in the plasma that have rapid association / dissociation characteristics (Laidley and Thomas, 1994, 1997) was used successfully for the identification of solubilized membrane progestin receptors (Zhu et al., 2003a,b). Detergents such as Triton X-100, digitonin, and CHAPS were effective in removing proteins from the membrane but resulted in high non-specific binding that had to be removed prior to radioreceptor assay. Only one of these detergents, Triton X-100 could be separated easily from the solubilized fraction by absorption using specialized polyabsorbent beads (Bio-Rad SM-2) and a filtration step. Therefore, Triton X-100 was used to solubilize the membrane proteins including mPR from the membrane, and the detergent was removed following the solubilization (Fig. 1). Moreover, due to the high absorption of Triton X-100 at OD 280 nm, any residual amounts of Triton X-100 that remained in the medium could be detected easily.

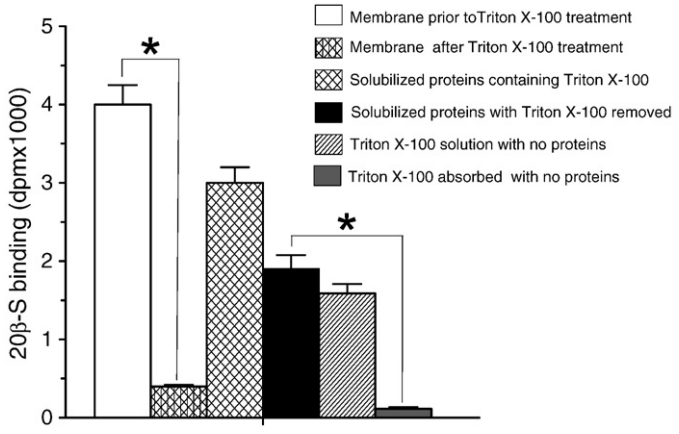

Fig. 1. Comparison of progestin (20β-S: 17α,20β,21-trihydroxy-4-pregnen-3-one) binding in membranes or solubilized proteins with or without the detergent. Membrane proteins of seatrout ovarian tissues were solubilized by stirring a purified membrane fraction with 12 mM Triton X-100 for 30 min at 4 °C, followed by removal of the detergent with polyabsorbent beads (Bio-Rad, SM2) by stirring at 4 °C for 2 h. *: the two treatments were significantly different analyzed by Student's *t*-test ($p<0.001$).

Large amounts of ovarian tissue were collected from spawning spotted seatrout, and various purification methods including gel filtration, ion exchange columns, and HPLC steps were attempted. However, the use of multiple purification steps caused a rapid loss of receptor binding activities in purified fractions, so that the original approach of using biochemical purification methods to obtain purified membrane progestin receptor protein for amino acid sequencing was abandoned. Instead, multiple approaches that included an initial biochemical purification step, followed by immunological identification and molecular screening were adopted. Following partial purification and concentration of solubilized membrane proteins, an ion exchange column (Pharmacia DEAE Sepharose CL-6B) was used to purify the mPR into highly homogeneous fractions, which were then concentrated (Zhu et al., 2003b), and used to immunize mice. Monoclonal antibodies were generated and selected based on their binding to the purified mPR fractions using Western blotting analyses. Then, a novel/rapid receptor capture assay was developed using the principles of ELISA and a receptor binding assay (Thomas et al., 2002), which enabled the identification of three monoclonal antibodies that had the capacity to capture the putative membrane progestin receptors. With the availability of multiple antibodies as tools, the first clone of the membrane progestin receptor (mPRα) was identified from a cDNA expression library generated from seatrout ovaries (Zhu et al., 2003b). Subsequently, orthologs of the mPR were identified in mammalian species and cloned from other model species including zebrafish and *Xenopus* (Zhu et al., 2003a).

5. mPRs—novel receptors coupled to G proteins in vertebrates

The mPRs are well conserved across a broad range of vertebrate species from fish to humans, having similar GPCR-like structures including seven transmembrane domains, and conserved cysteine residues for disulfide bonding (Zhu et al., 2003a; Thomas et al., 2004). Despite their similarities to GPCRs, the mPRs have been grouped in a unique receptor class called the progestin and adiponectin receptor (PAQR) family, which is based on seven transmembrane domains and

an uncharacterized UPF0073 motif (Tang et al., 2005). The PAQR family contains at least 11 unique members found across a broad range of organisms from *Eubacteria* to humans (Tang et al., 2005; Thomas et al., 2007). The mPRs and their PAQR receptor family are descendents of hemolysins, which are restricted to *Eubacteria* (Thomas et al., 2007). In contrast, phylogenetic analysis shows that typical GPCRs are direct descendents of bacterial rhodopsin, originating in *Archaebacteria*. This suggests that mPRs have evolved GPCR-like characteristics in parallel with typical GPCRs in eukaryotic organisms, and are an example of convergent evolution. To date mPR isoforms have demonstrated typical GPCR-like functions including characteristic orientation with the N-terminus on the outside of the cell and the C-terminus on the inside of the cell, coupling to heterotrimeric G proteins, and mediation of rapid nongenomic signaling (Zhu et al., 2003a; Thomas et al., 2007).

The mPRα and mPRβ share about 50% or greater sequence homology at the nucleotide level, not only to each other in the same species, but also across a broad range of species (Zhu et al., 2003a,b; Kazeto et al., 2005). The mPRγ is more divergent and shares only about 30% sequence homology to mPRα and mPRβ in various species and possibly mediates different functions. Northern blot analysis of human tissue extracts and real-time quantitative RT-PCR of fish tissue extracts have shown that the mPRα and mPRβ are generally co-localized and have high expression in the brain and reproductive tissues, whereas mPRγ is highly expressed in the lung, liver, kidney and fallopian tube (Zhu et al., 2003a; Nutu et al., 2007). However, all three receptor subtypes have been detected in human breast cancer cells and are likely co-expressed in a variety of other progestin target cells (Dressing and Thomas 2007). Recent studies suggest that mPRα and mPRβ mediate progestin signaling and functions in a variety of cell types and animal models, including induction of oocyte maturation in fish and amphibians (Zhu et al., 2003b; Tokumoto et al., 2006; Josefsberg Ben-Yehoshua et al., 2007), and sperm motility in fish (Thomas et al., 2005; Tubbs and Thomas, 2008). Progesterone signaling via mPRs has been demonstrated in human myometrial cells, breast cancer cells and lymphocytes (Karteris et al., 2006; Dressing and Thomas 2007; Thomas et al., 2007; Dosiou et al., 2008), and in rat and sheep reproductive tissues (Cai and Stocco 2005; Ashley et al., 2006). Taken together, these studies suggest a widespread involvement of mPRs in mediating nongenomic progestin actions in vertebrate target tissues.

Recombinant mPRα proteins of seatrout, goldfish, zebrafish, sheep and human, as well as mPRβ proteins from *Xenopus* and zebrafish, produced in eukaryotic expression systems, and mPRα, mPRβ, and mPRγ proteins of human and mouse produced in a prokaryotic expression system, have all been shown to specifically bind progestins (Zhu et al., 2003a,b; Ashley et al., 2006; Tokumoto et al., 2006; Josefsberg Ben-Yehoshua et al., 2007; Thomas et al., 2007). Studies have demonstrated that mPRα, the first identified and most studied form, mediates progestin-initiated actions in oocyte maturation (Zhu et al., 2003b; Tokumoto et al., 2006) and is also a likely candidate as an intermediary in some effects of progestins on sperm hypermotility, parturition, breast cancer, and immunoregulation (Thomas et al., 2005; Karteris et al., 2006; Dressing and Thomas, 2007; Tubbs and Thomas, 2008; Dosiou et al., 2008). The seatrout mPRα is localized on the oocyte membrane, its expression in late stage oocytes increases prior to oocyte maturation, and the receptor is hormonally upregulated by gonadotropin during oocyte 'priming' (Zhu et al., 2003b). The seatrout mPRα also signals in a manner consistent with previous reports on fish oocyte maturation (Nagahama 1997). Activation by the known MIS of the species, 20β-S, causes seatrout mPRα-mediated stimulation of the MAPK cascade and inhibition of cAMP production through a pertussis toxin sensitive, G_i coupled inhibitory pathway (Zhu et al., 2003b; Pace and Thomas. 2005; Thomas et al., 2007). Similar findings have been obtained with the zebrafish mPRα and mPRβ (Hanna et al., 2006). Both the mPRα and mPRβ are coupled to an inhibitory G_i protein in human myometrial cells which causes inhibition of adenylyl cyclase and a subsequent reduction in cAMP levels (Karteris et al., 2006). Overexpression of seatrout and zebrafish mPRα, and zebrafish mPRβ in cell lines has also been associated with increased progestin-induced MAPK activity (Zhu et al., 2003b; Hanna et al., 2006). These results provide strong evidence supporting mPRs as the MIS receptors in mediating oocyte maturation. However, the specific actions of mPRα and/or mPRβ, including possible interactions of the mPRs and cross-talk with other receptors including nPRs during oocyte maturation is currently unknown and requires further investigation.

The mPRγ has be shown to mediate progestin-induced oocyte transport, although direct evidence is lacking (Nutu et al., 2007). In addition, the coupling and signaling pathways of mPRγ have not been examined. Further studies are required to examine how each mPR isoform mediates signaling pathways and different functions in the broad spectrum of nonclassical progestin responses.

6. Progesterone receptor membrane components 1 and 2 (PGRMCs)

A putative progesterone binding protein was purified and partially sequenced from porcine microsomal membranes (Meyer et al., 1996). Based on the partial N-terminal sequence, the full-length putative progesterone binding protein was cloned from porcine vascular smooth muscle cells (Falkenstein et al., 1996). A rapid solubilization procedure (i.e. 1 min stirring at room temperature) was used to extract the membrane proteins from the microsomal membrane preparations using the zwitterionic detergent, CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate). The detergent was not removed during the subsequent purification and radioreceptor assay steps. Interestingly, the corresponding author (Dr. Yong Zhu) was unable to extract the mPR from seatrout oocyte membranes (>90% of the binding would be retained by the membrane) or assay binding effectively using this protocol.

The cloned progestin binding moiety was initially named progesterone binding protein, then membrane-associated progesterone receptor, and later was renamed as progesterone receptor membrane component 1 (PGRMC1). The PGRMC1 contains 194 amino acids, an N-terminal transmembrane domain (25–43aa), and a cytochrome *b*5-like or heme/steroid binding domain (72–135aa) (Mifsud and Bateman, 2002). Preliminary results suggest that PGRMC1 may influence the acrosomal reaction in mammalian sperm and the regulation of anti-apoptotic actions of progesterone in granulosa/luteal cells (Lösel et al., 2005; Peluso et al., 2008). Only limited studies have been conducted on the closely related PGRMC2. Interestingly, both PGRMC2 and PGRMC1 are expressed on human sperm and have been suggested to be involved in the progesterone-induced acrosomal reaction (Falkenstein et al., 1999; Lösel et al., 2005). However, progesterone-initiated signaling and receptor function through PGRMC1 or its homolog PGRMC2 have yet to be clearly demonstrated and thoroughly described. Sub-cellular fractionations of the proteins revealed an identical distribution of PGRMC1 with endoplasmic reticulum membrane markers (Nölte et al., 2000), although PGRMC1 has been reported on the plasma membranes of spontaneously immortalized granulosa cells (Peluso et al., 2006). In addition, PGRMC1 has been cloned independently by a number of groups and given a variety of additional names and ascribed functions other than those mediating the response to progestins. Other names for the PGRMC1 include adrenal inner zone antigen (IZA), VEMA, ratp28 and 25-Dx with functions as diverse as a regulation of water homeostasis in the brain, a heme binding protein, and a ventral midline antigen in regulating axon guidance in the mammals (Runko et al., 1999; Nölte et al., 2000; Meffre et al., 2005; Min et al., 2005). The protein is also expressed in the inner zones of the rat adrenal cortex, suggesting roles related to steroid synthesis or metal metabolism (Raza et al., 2001). Intriguingly, PGRMC1 shares homology and a conserved Cyt-b5

domain with neudesin, a putative neuron derived neurotrophic factor (Kimura et al., 2005; Kimura et al., 2006), which has no binding affinity for any steroids including progesterone, but instead has high affinity for hemin (Kimura et al., 2008). The conserved Cyt-b5 domain in the PGRMC1 is also a characteristic domain at the N-terminal region of fungal chitin synthases, and mammalian HERC2 (a potential E3 ubiquitin protein ligase and/or guanine nucleotide exchange factor) (Mifsud and Bateman, 2002; Kimura et al., 2008). These results support structural analyses that PGRMC1, HERC2, fungal chitin synthases, and neudesin all have a Cyt-b5 like heme binding domain (Mifsud and Bateman, 2002). Further studies on PGRMC1 mediation of rapid progestin signaling and functions are required to validate the protein as a functional receptor or a binding partner for progestins.

7. Nuclear progestin receptors (nPRs)

In addition to the well-characterized genomic mechanism of progestin action through nPRs investigated in detail over the past 30+ years, nPRs have been shown to mediate rapid nongenomic progestin signaling in several cell types, including immortalized breast cancer cells and amphibian oocytes (Bayaa et al., 2000; Tian et al., 2000; Faivre et al., 2005). In *Xenopus* oocytes and human breast cancer cells, over-expression of nPRs increase rapid intracellular nongenomic signaling in response to progestins resulting in activation of MAPK and cell cycle regulators such as cyclins (Bayaa et al., 2000; Tian et al., 2000; Faivre et al., 2005; Skildum et al., 2005; Boonyaratanakornkit et al., 2007). Two nPRs (XPR-1 and XPR-2) have been identified in *Xenopus* (Bayaa et al., 2000; Tian et al., 2000). XPR-2 is a shortened version very similar to XPR-1, with some coding mismatch in the A/B region of the receptor, potentially implying transcription from two separate loci as is the case for eel nPRs (Ikeuchi et al., 2002; Liu et al., 2005). Over expression of XPR-1 or XPR-2 accelerates progesterone-induced oocyte maturation and cell cycle re-entry, while XPR-1 antisense inhibits the process (Bayaa et al., 2000; Tian et al., 2000). Additionally, XPR-1 has been shown by Western blot analysis to be present in small quantities in oocyte membrane fractions and to interact with the activated form of PI3K, a plasma membrane-associated signaling protein (Bagowski et al., 2001). Interestingly, an almost identical function has been demonstrated for mPRβ in *Xenopus* oocytes, including oocyte membrane localization, progestin binding, and control of oocyte maturation (Josefsberg Ben-Yehoshua et al., 2007), suggesting a potential interaction between mPRs and nPRs. However, to date the involvement of nPR in mediating final oocyte maturation has not been demonstrated in any other species.

The classical human nPR-B appears to mediate progesterone-triggered activation of Src and downstream MAPK signaling in human breast cancer cells through direct binding between a proline-rich domain within the amino terminus of nPR-B and the SH3 domain of Src (Boonyaratanakornkit et al., 2001). However, the amino-terminal proline-rich motif identified in the human nPR-B is not conserved in several other model organisms such as mice, *Xenopus* or zebrafish. Therefore, it is unclear how nongenomic progestin signaling is mediated by nPRs in these species.

It is also unclear how the nPR associates with the membrane to mediate rapid cell surface-initiated progestin signaling. The nPRs have the typical nuclear steroid receptor structure with well defined A/B, DNA binding, hinge and ligand binding regions, but no defined transmembrane regions for association with the cell membrane. Recently, a palmitylation motif was identified within the ligand binding region of human nPRs and in other classical steroid receptors (Pedram et al., 2007). The post-translational addition of a fatty acylation may allow a small fraction of the nPRs to associate with the membrane and mediate membrane signaling. However, it is currently not known whether the recognition site conferring the palmitylation is conserved in other species. In vivo, only a small percentage (<5%) of nPR is detected at the plasma membrane (Bagowski et al., 2001). However, other groups have been unable to detect the membrane localization of the nPR in *Xenopus* (Bayaa et al., 2000; Tian et al., 2000; personal communication) despite their intensive efforts. To validate nPR as a receptor for mediating cell surface-initiated actions of progestin, further research is needed to demonstrate functionality and the conserved nature of motifs for cell membrane location and intracellular signaling.

8. Receptor interactions

Although the overlapping expression of mPRs and nPRs in tissues such as oocytes, the myometrium, and breast cancer cells suggest potential cross-talk between the two classes of progestin receptors, this has received little attention to date. Potential interactions between different progestin receptors may alter receptor signaling and function during nongenomic events such as oocyte maturation. Both mPRα and mPRβ are located at the oocyte membrane and both receptors have been implicated in progestin signaling leading to final oocyte maturation (Zhu et al., 2003b; Thomas et al., 2004; Hanna et al., 2006; Josefsberg Ben-Yehoshua et al., 2007). The mPRα and mPRβ are also co-localized in other tissues including T lymphocytes and myometrial cells, with elevated activity of the mPRα isoform (Karteris et al., 2006; Dosiou et al., 2008). Dimerization of the mPRs is possible as heterodimer or homodimer pairs, which may affect receptor signaling and ligand binding as exhibited by a number of GPCRs (Waldhoer et al., 2005; Franco et al., 2007). The nPRs are also known to dimerize in homo or heterodimer pairs that affect receptor function (Vegeto et al., 1993). The nPR-A can function as a dominant negative receptor to repress the transcriptional activity of nPR-B by hetero-dimerizing, subsequently inhibiting transcription of gene products (Vegeto et al., 1993). In addition, co-localization, interaction and cross-talk in signaling between mPRs and classical nPRs have been demonstrated in human myometrial cells in a recent study (Karteris

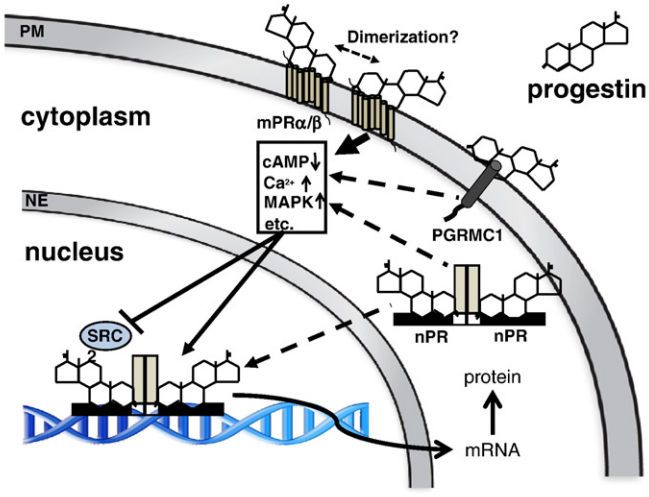

Fig. 2. Potential nongenomic and genomic signaling pathways of progestins via membrane progestin receptors (mPRs), progestin receptor membrane components (PGRMC) and nuclear progestin receptors (nPRs). In responses to progestin stimulus, rapid nongenomic progestin actions are initiated by mPRs or PGRMCs located at plasma membrane (PM). These responses include activation or inhibition of signaling intermediators such as cAMP, Ca^{2+}, and MAPK in target cells. Changes in these signaling intermediators can eventually lead to longer term transcriptional changes in the cell and regulation of nPR transcriptional activities via inhibition of nuclear receptor regulatory proteins such as steroid receptor coactivator 2 (SRC2). The nPR may also elicit rapid nongenomic responses from a membrane or cytoplasmic localization in addition to its well known transcriptional activity within the nucleus. In addition, there is potential for interaction of these various signaling pathways and receptors in order to fine-tune the final physiological response to progestins within the target cell. (NE: Nuclear envelope).

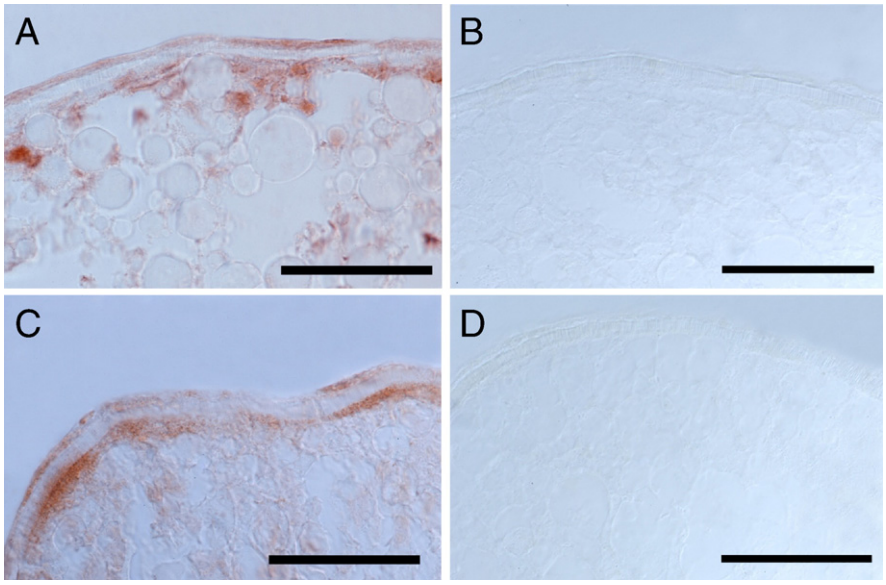

Fig. 3. Localization of zebrafish mPRα and mPRβ proteins in late vitellogenic oocytes (stage IV) by immunohistochemistry using specific zebrafish mPR antibodies (A: mPRα, C: mPRβ) and pre-immune serum (B: mPRα, D: mPRβ). The mPRα and mPRβ proteins appeared at or near the oocyte membrane and in the follicular cells surrounding the oocyte. Scale bar: 50 μm.

et al., 2006). The interaction between membrane and nuclear receptor signaling has been suggested based on downregulation of steroid receptor coactivator (SRC2) by mPRα in human myometrial cells (Karteris et al., 2006). These studies suggest that progestin hormones could potentially bind to and activate progestin receptors at multiple sites in target cells, initially binding to receptors on the cell surface and subsequently to intracellular receptors, concluding with binding to those in the nucleus (Fig. 2). Progestin binding to these multiple receptor classes may result in a complex temporal pattern of cellular changes, including rapid as well as long term responses, both of which are likely to be important for the coordinated hormone response of the cell. Progestin binding to multiple receptor classes may also result in complex interactions among their signaling pathways, some of which could act in concert and others in opposition, thereby eliciting a variety of cellular responses depending on the physiological status of the cell, the hormonal profile, and the subtypes of PR present. However, additional studies are required in order to confirm the interactions between the genomic and nongenomic pathways mediated through mPRs and nPRs in progestin target cells and to understand their importance in modulating the overall physiological responses of animals to progestins.

9. Current technological problems, controversies and future areas of research

There are several technical obstacles and controversies currently concerning the identities and functions of progestin receptors mediating rapid nongenomic actions, including their localization on cell membranes, difficulties in expressing recombinant progesterone membrane receptors in mammalian cell lines, and demonstrating their ability to bind progestins and mediate progestin signaling. Membrane localization of recombinant progestin receptors is necessary for investigating nongenomic signaling relevant for progestin actions such as induction of oocyte maturation, since there is clear evidence that the initial nongenomic steroid signaling processes in oocytes are initiated at the cell surface. Investigations of the expression and functions of membrane progestin receptors in their natural tissues/cells such as oocytes are necessary to confirm the results of functional studies with recombinant proteins in non-native cells such as mammalian cell lines. The zebrafish mPRα and mPRβ proteins are endogenously expressed at plasma membranes of oocytes (Zhu et al., 2003b; Fig. 3). However, the expression and localization of zebrafish mPRs in non-native cells (mammalian cell lines) are variable and depend on the constructs, tags, and expression systems employed (Fig. 4). Therefore, it is not surprising that different results on mPR expression and function have been obtained by different laboratories (Zhu et al., 2003b; Hanna et al., 2006; Krietsch et al., 2006). Matching the size of expressed recombinant mPR proteins in the mammalian cell lines with their predicted molecular weights is necessary to confirm the functional expression of the entire mPR protein, even if the experimental results have failed to show membrane localization of the mPR. Unfortunately, Brosens and coworkers (Krietsch et al., 2006) did not provide such evidence when they discussed their negative results for plasma membrane localization, binding and signaling of recombinant mPRs. Currently, the mechanisms regulating the trafficking of mPR proteins to the plasma membrane and their internalization are unknown. Using different tags and linking the tag at the N-terminal or C-terminal of zebrafish mPR affected its localization on the plasma membrane (Fig. 4; data not shown). Additionally, the

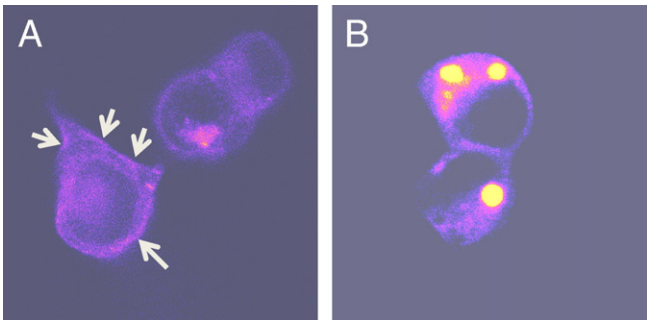

Fig. 4. Confocal images of HEK-293 cells transfected with C-terminal tagged zmPRβ-YFP (A) or N-terminal tagged YFP-zmPRβ (B) constructs. HEK-293 cells were transiently transfected with one expression vector each time. The cells were cultured over night in media with charcoal-stripped fetal bovine serum and imaged at 24 h after transfection. Fluorescent proteins were observed in plasma membrane of cells transfected with a C-terminal tagged zmPRβ-YFP construct (indicated by arrow), whereas fluorescent proteins were trapped inside the cells transfected with N-terminal tagged YFP-zmPRβ construct.

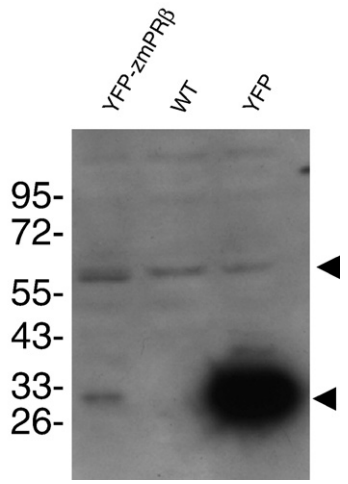

Fig. 5. Comparison of fluorescent protein (YFP) expression in cell lysates of wild type (WT) HEK-293 cells with no plasmid or cells transfected with YFP-zmPRβ or YFP expression plasmids analyzed by Western blotting. Arrowheads indicate YFP monomer and YFP dimer, respectively. Instead of an YFP-mPRβ fusion protein, wild type YFP was detected in cells transfected with the YFP-zmPRβ construct, possibly due to cleavage of YFP-mPRβ fusion protein.

fusion construct may be digested or degraded (Fig. 5) and toxic to the host cells (Fig. 6) in some cases. Therefore, failure to express mPRs or identify them on the plasma membrane in studies using fusion constructs such as mPR-fluorescent proteins need to be interpreted cautiously.

The nPRs have no well defined transmembrane domains and instead may use mechanisms such as palmitylation for association with cell membranes (Pedram et al., 2007), although a uniform mechanism for this location process across species has not been demonstrated. In contrast, mPRs and PGRMCs have distinct transmembrane structures and are localized to cell membranes under certain conditions. However, proper over-expression and subsequent analysis of function of mPRs and PGRMCs on cell membranes has proven difficult. Differing reports on mPR expression in transfected cells may be due to inhibition of expression by receptor tags or the unavailability of certain undefined accessory proteins in individual tissues or cell lines (Krietsch et al., 2006). For example, PGRMC-1 requires expression of its binding partner plasminogen activator inhibitor 1 RNA binding protein (PAIRBP1) to mediate proper functions as a progestin membrane receptor (Lösel et al., 2004, 2005). There may also be a similar variation in endogenous expression patterns of these receptors in different tissues due to variations in the availability of accessory proteins or processes regulating receptor expression. Many questions remain on the ability of each progestin receptor subtype to mediate nongenomic signaling. Human nPR-B possesses an identified polyproline motif that may allow association with signaling molecules (Boonyaratanakornkit et al., 2001), but the motif is not conserved in other model organisms such as mice, *Xenopus* or zebrafish. The mPRs associate with G_i inhibitory proteins, but the exact nature of the G protein/receptor interactions are

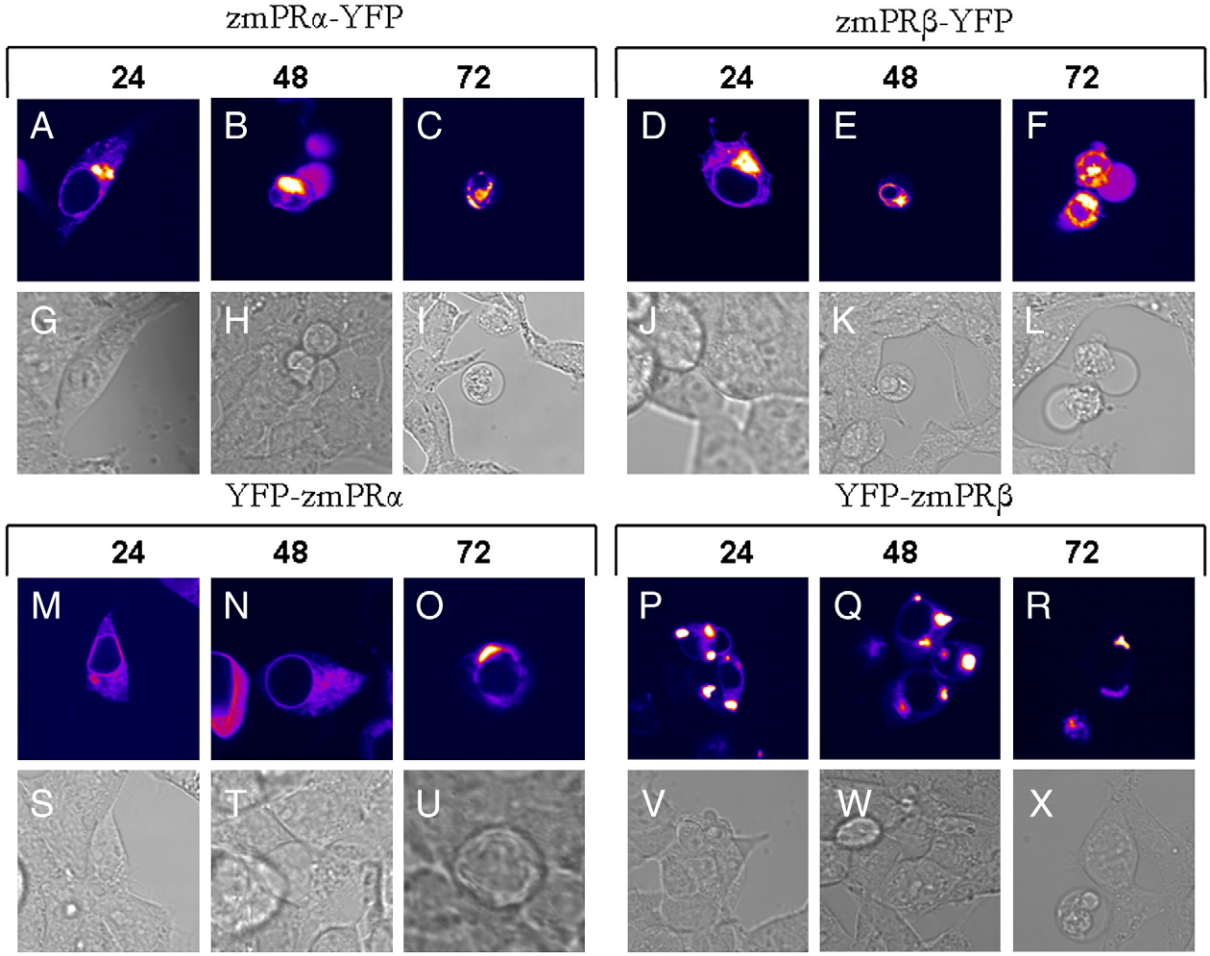

Fig. 6. Confocal and bright field images of HEK-293 cells transiently transfected with zmPRα-YFP (confocal A–C, bright field G–I), zmPRβ-YFP (confocal D–F, bright field J–L), YFP-zmPRα (confocal M–O, bright field S–U) and YFP-zmPRβ (confocal P–R, bright field V–X) at 24, 48 and 72 h after transfection. Abnormal cell shape (round) and cell death observed at 48 and 72 h after the transfection may be due to toxic effects of the constructs.

unknown since they are not typical GPCRs (Thomas et al., 2007). The PGRMCs have several potential Src homology domains for potential interactions with signaling molecules, but signaling directly through PGRMCs has not been demonstrated (Peluso et al., 2008). The precise functions and requirement of each of these progestin receptors in the wide array of nongenomic progestin actions that have been identified to date are unknown. Interestingly, the mPRs and nPRs show varying amounts of overlapping expression and potential interaction in a number of nongenomic actions including oocyte maturation, parturition, and breast cancer signaling, suggesting possible cross-talk and interaction between these different classes of PRs.

10. Concluding remarks

Oocyte maturation is an excellent model for studying nongenomic actions of progestins. In order to obtain a complete understanding of progestin receptor-mediated function during oocyte maturation it will be necessary to determine the signaling pathways initiated through each of these receptors and their respective functions in the control of this process. Information will also be required on the changes in the expression of these receptors, and their hormonal regulation preceding and during oocyte maturation. It will be important to assess the interactions between the two progestin receptor classes (mPRα/mPRβ/nPR-A/nPR-B), and verify the requirement for each progestin receptor in mediating nongenomic progestin induction of oocyte maturation by loss and gain of function approaches. Previous studies suggest that both mPRs and nPRs have a role in oocyte maturation (Bayaa et al., 2000; Tian et al., 2000; Zhu et al., 2003b). However, additional studies are required to demonstrate direct roles for each of the receptors in mediating MIS induction of oocyte maturation *in vivo*. Additional information is also needed on how multiple receptors mediate progestin-induced signaling pathways during oocyte maturation. Therefore, a better understanding of functions mediated by mPRs and nPRs in oocyte maturation has great potential to advance our general understanding of progestin-mediated nongenomic signaling in vertebrate reproductive tissues.

Acknowledgments

This work was supported in part by the National Science Foundation Grant IBN-0315349 (Y.Z.) East Carolina University Research and Creative Activity Grant 2003-2010 (Y.Z.), East Carolina University Thomas Harriot College of Arts and Sciences Research Award (Y.Z), East Carolina University Division of Research and Graduate Studies Research 2007 Development Award (Y.Z.), grants from Cyttron in the Besluit Subsidies Investeringen Kennisinfrastructuur Program (M.S), and by a grant in the SmartMix program.(M.S). We also like to thank Joost Broekhuis for the technical assistance.

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