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Plasma Membrane Destination of the Classical *Xenopus laevis* Progesterone Receptor Accelerates Progesterone-Induced Oocyte Maturation

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Abstract *Xenopus laevis* oocyte maturation is induced by the steroid hormone progesterone through a non-genomic mechanism initiated at the cell membrane. Recently, two *Xenopus* oocyte progesterone receptors have been cloned; one is the classical progesterone receptor (xPR-1) involved in genomic actions and the other a putative seven-transmembrane-G-protein- couple receptor. Both receptors are postulated to be mediating the steroid-induced maturation process in the frog oocyte. In this study, we tested the hypothesis that the classical progesterone receptor, associated to the oocyte plasma membrane, is participating in the reinitiation of the cell cycle. Addition of a myristoilation and palmytoilation signal at the amino terminus of xPR-1 (mp xPR-1), increased the amount of receptor associated to the oocyte plasma membrane and most importantly, significantly potentiated progesterone-induced oocyte maturation sensitivity. These findings suggest that the classical xPR-1, located at the plasma membrane, is mediating through a non-genomic mechanism, the reinitiation of the meiotic cell cycle in the *X. laevis* oocyte. J. Cell. Biochem. 99: 853–859, 2006.

Key words: *Xenopus* oocyte; progesterone receptor; progesterone

Steroid hormones are traditionally known to mediate their biological activities via nuclear receptors by regulating gene transcription. In the last decade however, many steroid-induced signaling events have been reported to occur via membrane receptors, which are independent of transcription. This new signaling mechanism has been called "non-genomic" steroid action and is characterized by a faster time of action, from seconds to minutes, and to be insensitive to transcription inhibitors [Hammes, 2003; Losel and Wehling, 2003; Edwards, 2005]. A long list of examples of rapid non-genomic actions induced by different components of the steroid hormone family, which are acting in a wide variety of systems, have been reported. Among them, perhaps the best-characterized example of progesterone non-genomic action that is mediated by a membrane receptor and does not require transcription, is the induction of *Xenopus laevis* oocyte maturation [Ferrel, 2005].

Xenopus laevis Stage VI oocytes are arrested at the G2/M transition of meiosis I and under progesterone stimulus, which is secreted by the oocyte surrounding follicular cells, their cell cycle is reinitiated and arrested again in metaphase II, until fertilization. This signaling pathway, called oocyte maturation, at a very early step induces inhibition of adenylyl cyclase (AC), with the concomitant drop of intracellular cAMP levels and PKA inhibition. Recently, Gαs and Gβγ have been shown to be playing an

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important role as positive regulators of oocyte AC and as a consequence of that, of keeping the oocyte in an arrested state. Overexpression of Gαs and Gβγ were able to block progesterone-induced maturation and their depletion, to accelerate cell cycle progression [Gallo et al., 1995; Lutz et al., 2000; Sheng et al., 2001; Romo et al., 2002; Kalinowski et al., 2004]. Furthermore, by interfering with AC activation, through microinjection of specific Gβγ-AC blocking peptides or by expression of Gβγ inactive mutants, oocyte progesterone-induced maturation was significantly potentiated [Guzmán et al., 2004; Sheng et al., 2004]. All these data indicate that Gαs, Gβγ, and AC are the molecular components that maintain elevated intracellular oocyte cAMP levels and hence oocyte arrest. However, the mechanism involved in progesterone-induced AC inhibition and the identity of the oocyte membrane progesterone receptor, are still open questions and a matter of debate.

Earlier observations have shown that progesterone inhibits AC in a GTP-dependent and pertussis toxin-insensitive manner [Jordana et al., 1981; Sadler and Maller, 1981; Goodhardt et al., 1984; Olate et al., 1984; Sadler et al., 1984], through a mechanism that involves the slowing of the GDP/GTP exchange rate of oocyte G proteins [Sadler and Maller, 1982]. In addition, great efforts have been done by different groups to try to identify the oocyte plasma membrane progesterone receptor and several putative receptors have been reported. By using a synthetic progestin (R5020), Sadler and Maller [1982] were able to photoaffinity label an oocyte surface protein of 110,000 Da. On the other hand, the group of Baulieu, using the same technique, reported a progesterone receptor of 30,000 Da [Blondeau and Baulieu, 1984]. Almost two decades later, the *X. laevis* classical progesterone receptor (xPR-1) was cloned and proposed to be participating through a non-genomic mechanism in the rapid events that mediate oocyte maturation [Bayaa et al., 2000; Tian et al., 2000]. Recently, an other putative progestin G-protein-coupled-receptor from human and other vertebrates, including *Xenopus*, was cloned and claimed to be the real membrane progesterone receptor that was inducing oocyte maturation [Zhu et al., 2003a,b].

From all the progesterone receptors described, there is important experimental evidence that strongly supports the idea that the classical xPR-1 is mediating the non-genomic progesterone action. First, overexpression of xPR-1 accelerates progesterone-induced maturation [Bayaa et al., 2000; Tian et al., 2000]. Second, injection of anti-sense oligonucleotides against xPR-1 almost ablate maturation in response to progesterone and, most importantly, microinjection of xPR-1 mRNA into the anti-sense treated oocytes restored their ability to respond to progesterone [Tian et al., 2000]. Third, xPR-1 has been shown by Western blot analysis to be present in small quantities in oocyte membrane fractions [Bagowski et al., 2001] and to interact with the activated form of PI3K, a plasma membrane associated protein. However, the principal caveat to this receptor is to understand how xPR can associate to the plasma membrane, since no lipid modifications or transmembrane regions have been reported for this receptor [Maller, 2001].

In order to demonstrate that the classical xPR-1, associated to the plasma membrane, is capable of inducing the non-genomic progesterone signaling, myristoylation and palmitoylation signals were added at the amino terminus of xPR-1 (mp-xPR-1), and oocyte maturation was analyzed in response to the steroid [Resh, 1999]. Here we report that mp-xPR-1 is fated to the plasma membrane and is significantly more potent in inducing progesterone-induced maturation than wild-type xPR.

MATERIALS AND METHODS

xPR-1 Cloning and Construction of pCS2+ Recombinant Vectors

The classical *X. laevis* progesterone receptor xPR-1 (Gene Bank accesion AF279335) was cloned by RT-PCR from Stage VI oocytes total RNA, using specific oligonucleotides. Wild-type xPR-1 was cloned using the sense primer 5′CCCGAATTCAAATGGAGGAGATAAGTC-GAGGAGATAAGTCAGACACCT3′ and the reverse primer 5′CCCCTCGAGTCACTTT TT GTG AAACACAAGTGGTTTTACCATCCCA GCT3′. Miristoylated and palmitoylated xPR-1 (mp-xPR-1), was obtained with the sense primer 5′CCCGAATTCCACG**ATGGGCTGT ATCAAGAGCAAGGGGAAA**GAGGAGATA AGTCAGACACCT-3′ and the previously described reverse primer. The *Eco*RI and *Xho*I restrictions sites that were incorporated in each oligonucleotide are shown underlined and the dual lipid modification signal is shown in

bold. xPR-1 and mp-xPR-1 amplified fragments were purified and subcloned into the *Eco*RI/*Xho*I sites of the pCS2+ vector (kindly donated by Dr. Roberto Mayor, Department of Anatomy and Development Biology, University College London, UK). Finally, the corresponding cDN As were excised from pCS2+ with *Eco*RI and *Xba*I and subcloned into pAGA2 vector.

In Vitro Transcription

xPR-1/pAGA2 and mp-xPR-1/pAGA2 were digested with *Sph*I and 1 μg of each linearized DNA was used as template for in vitro transcription with T7 RNA polymerase, in a volume of 20 μl, according to the instructions contained in the mMessage mMachine kit manual (Ambion, Austin, TX). The corresponding mRNAs were resuspended in RNase free sterile water, quantified by absorbance at 260 nm and stored at −80°C. The size and quality of the mRNAs was analyzed through 1.5% agarose gel electrophoresis and visualized with ethidium bromide and short UV light.

In Vitro mRNA Translation

The in vitro translation was performed as described previously [Olate et al., 1988; Antonelli et al., 1994], with some modifications. The translation mixture (10 μl) contained 1 μg of xPR-1 or mp-xPR-1 mRNA, 20 mM KCl, 20 μM of a non-radioactive aminoacid mixture without methionine, 7 μl of reticulocyte lysate, and 20 μM [^{35}S]methionine (18.2 μCi). The mixture was incubated at 30°C for 60 min and labeled proteins were analyzed on 10% SDS–polyacrylamide gel electrophoresis followed by autoradiography.

Oocytes Preparation

Adult *X. laevis* females, mantained with constant 12 h light and 12 h dark periods at 18°C, were anesthetized by hypothermia and pieces of ovary were surgically removed. Three grams of ovary tissue was separated in small pieces and incubated in a collagenase (Sigma, type I) solution (2% collagenase, 0.1% soybean trypsin inhibitor, and 0.1% bovine serum albumin) in 1× MR buffer (100 mM NaCl, 1.8 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 5 mM Na-HEPES, pH 7.8) at room temperature for 1 h 45 min. The collagenase treated oocytes were washed with 500 ml of 0.1% BSA and 100 mM KH_2PO_4, pH 6.5 and sorted for defolliculated Stage VI oocytes under a stereoscope. Oocytes were kept at 18°C on agarose dishes in oocyte culture medium (OR3) (50% Leibovitz's L-15 medium, 100 μg/ml gentamycin, and 15 mM HEPES pH 7.55) and used within 1 day after isolation. Maturation was induced by incubation with 0.5–1 μM progesterone and GVBD was scored by the appearance of a white spot in the animal hemisphere and confirmed after 10% TCA fixation. Oocyte microinjections were done in 1× MR medium and microinjected oocytes were incubated between 18–48 h at 18°C in OR3 medium before GVBD was scored or MAPK assay was performed.

Xenopus Oocytes Microinjection

Defolliculated Stage VI oocytes were kept at 18°C in agarose Petri dishes in oocyte culture medium OR3 (50% Leibovitz's L-15 medium, 100 μg/ml gentamycin, and 15 mM HEPES pH 7.55) and used within 1 day after isolation. Oocytes were microinjected with 30–50 nl of RNase free sterile H_2O containing the indicated amounts of xPR-1 or mp-xPR-1 mRNAs. Oocytes were incubated for 18 h at 18°C in OR3 medium before progesterone addition.

Oocyte Extract and Membrane Fraction Preparation

Stage VI oocyte extracts were prepared as described by Romo et al. [2002]. Briefly, oocytes were homogenized at 4°C by forcing them to pass through a pipetteman yellow tip in 8 μl of lysis buffer per oocyte, containing 100 mM K^+-glutamate, 10 mM EGTA, 3 mM $MgCl_2$, 20 mM Hepes, pH7.2, 10 μg/ml aprotinin, 12.5 μg/ml leupeptin, and 100 μg/ml of soybean trysin inhibitor. The homogenate was centrifuged three times at 325*g* at 4°C for 2 min and the supernatant was removed under light microscope and stored at −80°C. A more purified membrane fraction from Stage VI oocytes was prepared as described by Olate et al. [1984] with modifications. Oocytes were homogenized at 4°C in one volume of a solution containing 10 mM Hepes pH 8.0, 83 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF, 12.5 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μg/ml of soybean trysin inhibitor using a glass Dounce homogenizer. After 10 strokes with pestle A and 10 strokes with pestle B, the homogenate was centrifuged three times at 800*g* for 5 min in order to eliminate the pigment. The final supernatant was recentrifuged at 20,000*g* for 25 min and finally at 100,000*g* for 2 h at 4°C to obtain a

particulate fraction. The pellet represents an enriched oocyte plasma membrane fraction which was stored at −80°C until needed. Protein concentration was determined using the Bradford method.

Transfection of Cos-7 Cells and Total Cell Extract Preparation

Cos-7 cells were cultured in 100 mm plastic petri dishes containig DMEM-10% FBS, to a 50% cell density. Cells were washed three times with DMEM without serum and transfected with 5 μg of the following vectors: pCDNA3.1+, pCDNA3.1+/xPR-1 or pCDNA3.1+/mp-xPR-1 in a mixture containing DMEM and lipofectamine 2000 (Invitrogen). Cells were incubated at 37°C for 6 h with the transfection mixture and the media replaced with DMEM-10% FBS and incubated for 24–48 h at 37°C in a air- 5% CO_2 atmosphere. Cells were then lysed in a lysis buffer containing HEPES 10 mM, pH 7.9; DTT 1 mM; NaCl 100 mM; NP40 0.5%; PMSF 0.5 mM; aprotinin 1 μg/ml, and leupeptin 1 μg/ml and centrifuged at 4°C for 10 min at 10,000*g*. The supernatant was stored at −80°C.

Anti-xPR Antibody

Polyclonal antibodies against the amino terminal of the *Xenopus* progesterone receptor xPR-1 were raised in rats by injection of a purified GST-xPR-1 fusion protein, containing the first 371 aminoacids of the receptor. Briefly, glutation agarose purified GST-xPR-1 protein was separated trough SDS–PAGE and the corresponding band, excised from the gel according to the protocol described by Cozzani and Hartmann [1980], used as antigen to immunize Sprague–Dawley rats, following the protocol described by Nualart et al. [1991]. Blood samples (3 ml each) were collected under anesthesia by puncture of the retrocular sinus and the serum stored at −20°C.

Immunoblotting

Samples of transfected COS-7 cells, oocyte extracts, and enriched plasma membrane fractions were run on SDS–PAGE, transferred to Hybond ECL membranes (Amersham) and blocked with a buffer containing 10 mM Tris-HCl, pH 7.4; 100 mM NaCl; 0.05% Tween 20, and 5% non-fat milk. The blot was incubated for 1–4 h at 4°C with the primary antibody and after with horseradish peroxidase-conjugated secondary antibody. Anti-xPR-1 antibody was used at a dilution of 1:5,000 and peroxidase labeled anti-rat IgG at a dilution of 1:10,000 and developed with the Western Lightning Chemiluminescence kit (Pelkin Elmer, Boston, MA).

RESULTS

xPR-1 Is Expressed in *Xenopus* Oocytes and Migrates as a Protein of 100 kDa

In order to identify xPR-1 in *X. laevis* oocyte extracts, inmunoblot analysis with commercial antibodies raised against the human progesterone receptor were performed. Many non-specific bands were observed and none of them corresponded to xPR-1 (data not shown). Therefore, a specific antibody against the N-terminal half of xPR-1 was produced, as described in Materials and Methods, which detected only one 100 kDa protein. This band in water-injected oocyte extracts was in very low amounts, but increased many folds in xPR-1 mRNA injected oocytes (Fig. 1), confirming that the 100 kDa band detected by our antibody, corresponds to xPR-1. The higher molecular weight observed for mp-xPR-1 (108–110 kDa) was expected, and constitutes an indication that the lipid post-translational modification was introduced into the receptor. Also the low endogenous expression of xPR-1, detected in total *Xenopus* oocytes extracts, agrees with the results obtained by other groups [Bayaa et al., 2000; Tian et al., 2000].

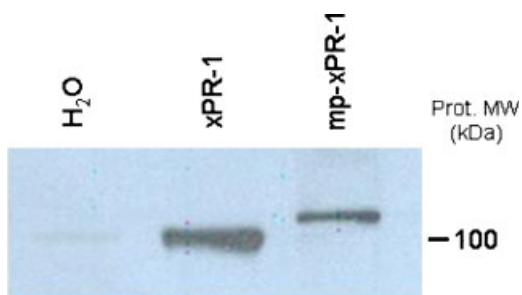

Fig. 1. Immunoblot analysis of xPR-1 and mp-XPR-1 expression in Stage VI oocytes. After microinjection, oocytes were incubated for 18–24 h at 18°C in OR3 medium and then lysed and total oocyte extract prepared as described in Materials and Methods. Protein (70 uf) from each group of oocytes was subjected to SDS–PAGE and then blotted using the anti-xPR antibody at a dilution of 1:5,000. H_2O: oocytes microinjected with H_2O; xPR-1: oocytes injected with 1 ng of wild-type xPR-1 mRNA; mp-xPR-1: oocytes microinjected with 1 ng of mp-xPR-1 mRNA.

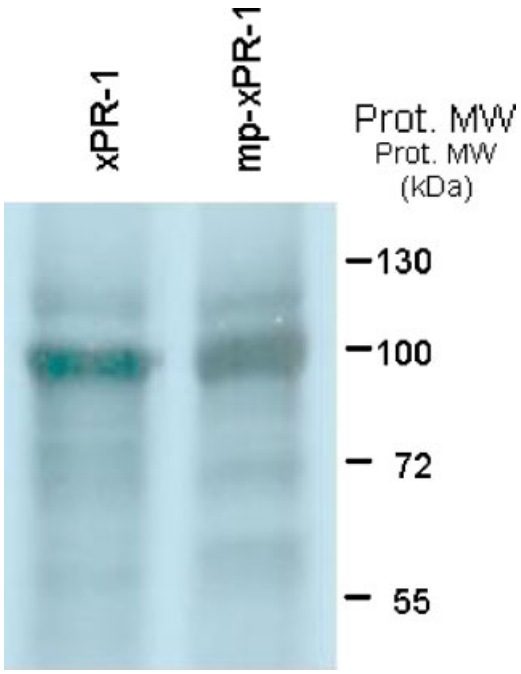

Fig. 2. In vitro translation of xPR-1 and mp-xPR-1 mRNAs. The in vitro translation was done as described in Materials and Methods using 1 μg of the respective mRNAs templates. The reticulocyte lysate mixture was subjected to SDS–PAGE electrophoresis and the resolved radioactive proteins were detected by autoradiography. xPR-1: in vitro translation of xPR-1 mRNA; mp-xPR-1: in vitro translation of mp-xPR-1 mRNA.

Since there was a considerably difference between the theoretical (82 kDa) and experimental molecular weight for xPR-1 (100 kDa), the size of the in vitro translated xPR-1 and mp-xPR-1 was determined. As shown in Figure 2, both xPR-1 and mp-xPR1 mRNAs, generated proteins of 100 kDa, which corresponds to the molecular weight observed in oocyte immunoblot analysis (Fig. 1). The difference in size obtained for mp-xPR-1 in both expression systems, is probably due to the absence of lipid modification in the in vitro translation system, since post-translational modifications do not take place efficiently in this expression system.

To further characterize the specificity of the xPR-1 antibody and to confirm the size of the steroid receptor, the cDNAs encoding xPR-1 and mp-xPR-1 were expressed in Cos-7 cells and the corresponding cell lysates analyzed by Western blot. A very similar result was obtained when both receptors were expressed in Cos-7 cells or in *Xenopus* oocytes (compare Figs. 1 and 3), therefore we can conclude that our antibody recognizes specifically xPR-1 and that it migrates in SDS–PAGE gel as a protein of 100 kDa.

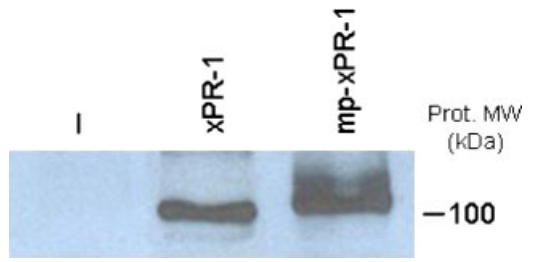

Fig. 3. Immunoblot analysis of Cos-7 cells transfected with xPR-1 and mp-xPR-1. After transfection the cells were cultured for 24 h and then lysed and total cell extract was prepared as described in Materials and Methods. Total cell extract protein (50 uf) was subjected to SDS–PAGE and then blotted using the anti-xPR antibody at a dilution of 1:5,000. –: COS-7 cells transfected with empty vector pCDNA3.1+; xPR-1: Cos-7 cells transfected with the recombinant vector pCDNA3.1+/xPR-1; mp-xPR-1: Cos-7 cells transfected with the recombinant vector pCDNA3.1+/mp-xPR-1.

mp-xPR-1 Associates to the Oocyte Plasma Membrane and Accelerates Progesterone-Induced Maturation

The proposal that the classical xPR is mediating oocyte maturation through a non-genomic mechanism that involves AC inhibition, implicates that the steroid receptor must be acting at the plasma membrane. To test this hypothesis, we decided to artificially bind xPR-1 to the oocyte plasma membrane, by the addition of a dual lipid post-translational modification signal (palmytoilation and myristoilation) at its amino terminus. We expected that oocytes overexpressing mp-xPR-1 would show increased levels of membrane bound receptor and a higher sensitivity to respond to progesterone in inducing oocyte maturation.

Indeed, as shown in Figure 4, mp-xPR-1 was distributed mainly in the membrane fraction compared to xPR-1 and more important than that, overexpression of mp-xPR-1 was able to accelerate progesterone-induced GVBD in a far more dramatical way than xPR-1. Figure 5 shows that 0.1 μM progesterone, a normally suboptimal steroid concentration to induce oocyte maturation, produced 80% GVBD in mp-xPR-1 overexpressing oocytes, compared to 30% observed in water or xPR-1 microinjected oocytes. These results indicate that xPR-1 is indeed acting at the oocyte plasma membrane, inducing through a non-genomic mechanism, progesterone-induced maturation.

DISCUSSION

The reported data demonstrate that endogenous xPR-1 is expressed in *Xenopus* oocytes in

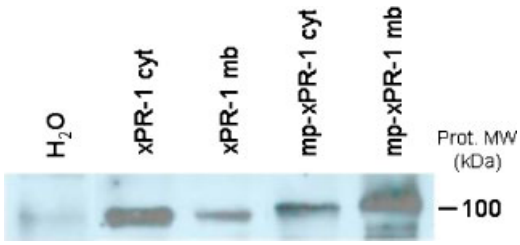

Fig. 4. Subcellular distribution of xPR-1 and mp-xPR-1 expressed in Stage VI *Xenopus* oocytes oocytes. After microinjection, oocytes were incubated for 18–24 h at 18°C in OR3 medium and then lysed and total oocyte extract prepared as described in Materials and Methods. Representative amounts of protein from each fraction, corresponding to 30 oocytes were subjected to SDS–PAGE and then blotted using the anti-xPR antibody at a dilution of 1:5,000. H_2O: oocytes microinjected with H_2O; xPR-1 cyt: cytosol fraction of oocytes injected with 1 ng of the wild-type xPR-1 mRNA; lxPR-1 mb: membrane fraction of oocytes injected with 1 ng of the wild-type xPR-1 mRNA; mp-xPR-1 cyt: cytosol fraction of oocytes microinjected with 1 ng of the mp-xPR-1 mRNA; mp-xPR-1 mb: membrane fraction of oocytes microinjected with 1 ng of the mp-xPR-1 mRNA.

low levels and that it migrates as a 100 kDa protein in SDS–PAGE gel. The size of the receptor was greater than the predicted molecular mass (82 kDa), which is probably due to anomalous migration of the protein or to post-translation modifications. This 100 kDa mass differs with the molecular weights reported for xPR-1 by other groups [Bayaa et al., 2000; Bagowski et al., 2001] but we are confident that our antibody is recognizing specifically xPR-1,

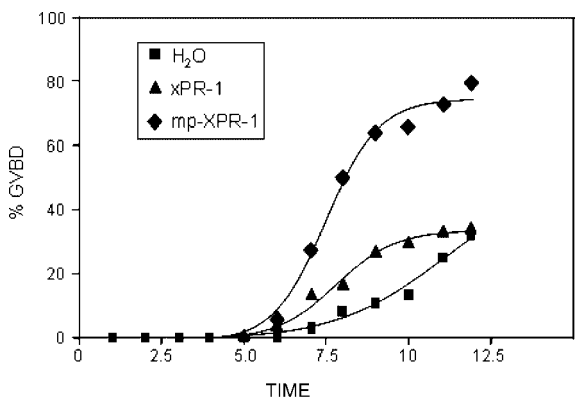

Fig. 5. Effect of xPR-1 and mp-xPR-1 overexpression in progesterone-induced *Xenopus* oocyte maturation. Stage VI oocytes were injected with H_2O (■), 1 ng of the xPR-1 mRNA (▲), or the mp-xPR-1 mRNA (◆), incubated for 18–24 h in OR3 medium and then incubated with 0.1 μM progesterone. Oocytes were scored for GVBD at the indicated times after progesterone addition. This experiment was repeated three times with similar results and a group of 100 oocytes was used for each condition.

since expression of the receptor in three different systems: microinjection of xPR-1 mRNA into *Xenopus* oocytes, transfection of Cos-7 cells with the xPR-1 cDNA, and in vitro translation of xPR-1 mRNA with reticulocyte lysate, originated a protein of the same size (100 kDa) as the one detected in *X. laevis* oocyte extracts.

In order to prove that the classical xPR-1 is mediating the induction of *X. laevis* oocyte maturation, it is very important to demonstrate its membrane localization. With our antibodies, we were unable to detect endogenous progesterone receptor associated to the oocyte membrane fraction, unless it was overexpressed. We believe that the sensitivity of the antibodies was not enough to detect the small quantities of xPR-1 that are associated to the oocyte membrane. According to previous work, approximately 3×10^8 high affinity progesterone binding sites are present in a *Xenopus* oocyte [Lindsey et al., 2000], and assuming a molecular weight of 100 kDa, a mass of 50 pg of membrane associated xPR-1 should be contained in each oocyte. Therefore, the total amount of membrane xPR-1 present in 30 oocytes, which was used in our Western analysis, should be approximately 1.5 ng. This quantity is in the limit of detection of the immunoblot technique, therefore we believe that a more sensitive antibody or technique is needed to detect xPR-1 associated to the membrane.

In order to proof that xPR-1 is acting at the plasma membrane of the oocyte, we decided to artificially introduce a lipid post-translational modification signal at the amino terminus of the receptor and look for its capacity to accelerate oocyte maturation. As expected, the addition of myristoilation and palmytoilation signals to xPR-1, significantly modified its intracellular distribution, increasing several times the proportion of receptor associated to the plasma membrane. However, the most important result was that increased amounts of membrane-associated xPR-1 also increased *Xenopus* oocytes sensitivity to progesterone. This observation further supports the idea that xPR-1, localized at the plasma membrane, is responsible for inducing progesterone non-genomic action in the *Xenopus* oocyte.

A question that still remains unanswered, is to understand the mechanism by which xPR-1 associates to the oocyte plasma membrane and inhibits AC. Further work will be necessary in the future to resolve this intriguing question.

In summary, our studies provide new evidence for the involvement of the classical xPR-1 in *X. laevis* oocyte maturation and shows that its non-genomic action is performed associated to the plasma membrane.

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