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A role for GPRx, a novel GPR3/6/12-related G-protein coupled receptor, in the maintenance of meiotic arrest in *Xenopus laevis* oocytes

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ABSTRACT

Progesterone-induced *Xenopus laevis* oocyte maturation is mediated via a plasma membrane-bound receptor and does not require gene transcription. Evidence from several species suggests that the relevant progesterone receptor is a G-protein coupled receptor (GPCR) and that a second receptor–GPR3 and/or GPR12 in mammals–tonically opposes the progesterone receptor. We have cloned a novel *X. laevis* GPCR, GPRx, which may play a similar role to GPR3/GPR12 in amphibians and fishes. GPRx is related to but distinct from GPR3, GPR6, and GPR12; GPRx orthologs are present in *Xenopus tropicalis* and *Danio rerio*, but apparently not in birds or mammals. *X. laevis* GPRx is mainly expressed in brain, ovary, and testis. The GPRx mRNA increases during oogenesis, persists during oocyte maturation and early embryogenesis, and then falls after the midblastula transition. Microinjection of GPRx mRNA increases the concentration of cAMP in oocytes and causes the oocytes to fail to respond to progesterone, and this block is reversed by co-injecting GPRx with morpholino oligonucleotides. Morpholino injections did not cause spontaneous maturation of oocytes, but did accelerate progesterone-induced maturation. Thus, GPRx contributes to the maintenance of G2-arrest in immature *X. laevis* oocytes.

Introduction

Fully grown, immature oocytes spontaneously arrest during the first meiotic prophase. Oocytes are released from this arrest and develop into fertilizable eggs through a process termed meiotic maturation. In vertebrate oocytes, the hallmarks of meiotic maturation include the resumption of meiosis I, which includes germinal vesicle breakdown (GVBD), chromosome condensation and spindle formation; the completion of meiosis I, with the extrusion of the first polar body; a brief period between meiosis I and meiosis II during which S-phase is blocked; and finally arrest in metaphase II because of cytostatic factor (CSF) activity. Meiosis II is completed after a mature oocyte is ovulated, at which point it becomes an egg, and capable of being fertilized (Ferrell, 1999).

In *Xenopus laevis* oocytes, maturation is triggered by the ovarian hormone progesterone. Progesterone acts through a non-classical plasma membrane receptor (Karaiskou et al., 2001; Maller, 1985) and causes a transient decrease in cAMP levels. This decrease in cAMP appears to be a key early event (Cork et al., 1990; Maller et al., 1979b); maturation can be blocked by an increased level of cAMP or by activation of PKA (Daar et al., 1993; Huchon et al., 1981; Masui and Markert, 1971), and maturation can be induced in the absence of progesterone by expression of a cAMP phosphodiesterase (Andersen et al., 1998). The decrease in cAMP is generally thought to initiate maturation, but it may also affect the positive feedback loops that ultimately bring about full activation of p42 MAPK and CDK1 just prior to GVBD (Duckworth et al., 2002).

Several groups have demonstrated that the classical progesterone receptor has non-genomic effects that contribute to oocyte maturation (Bagowski et al., 2001; Bayaa et al., 2000; Boonyaratanakornkit et al., 2001; Liu et al., 2005; Tian et al., 2000). In addition, progestins can be metabolized to androgens in oocytes (Lutz et al., 2001), and the classical androgen receptor may contribute non-genomic effects to oocyte maturation (Evaul et al., 2007; Hammes, 2004; Lutz et al., 2001). However, recent evidence suggests that one or more G-protein coupled receptors also play critical roles in the process. The main candidate for the relevant progesterone receptor is XmPRβ (Josefsberg Ben-Yehoshua et al., 2007), a *X. laevis* homolog of a membrane-bound progestin receptor originally cloned from fish oocytes (Zhu et al., 2003). XmPRβ and its close relatives constitute a subgroup of the PAQR proteins, a family of evolutionarily ancient seven-transmembrane proteins only distantly related to the other members of the GPCR superfamily (like the well-studied rhodopsin-like GPCRs, the Frizzled-like seven-transmembrane proteins, and so on) (Fredriksson et al., 2003; Tang et al., 2005; Thomas et al., 2006). As such they are only distantly related to GPR30, the membrane-bound estrogen receptor (Filardo and Thomas, 2005; Manavathi and Kumar, 2006;

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Prossnitz et al., 2007), which is a member of the chemokine receptor cluster of the γ-group of rhodopsin-like GPCRs.

Microinjection of XmPRβ antibodies blocks progesterone-induced maturation, and overexpression of XmPRβ potentiates the effects of progesterone (Josefsberg Ben-Yehoshua et al., 2007). The fish homolog of XmPRβ acts through a pertussis-toxin sensitive G_i protein to inhibit cAMP production, and so it might be expected that progesterone-induced *X. laevis* oocyte maturation would be sensitive to pertussis toxin as well. However, although pertussis toxin reportedly inhibits or slows down oocyte maturation in response to progesterone, it does not affect adenylyl cyclase inhibition by progesterone in isolated oocyte membranes (Sadler et al., 1984). Also, unlike typical G-protein-mediated processes in which the rate of GTP exchange onto α subunits increases with receptor stimulation, applying progesterone to isolated oocyte membranes decreases the rate of GTP exchange with the membranes (Sadler and Maller, 1985). These unusual properties raise the possibility that XmPRβ might act by inhibiting G_s rather than activating G_i.

Consistent with this idea, Jaffe and colleagues demonstrated, first in frog (Gallo et al., 1995), later in mouse (Mehlmann et al., 2002), and most recently in fish (Kalinowski et al., 2004), that injection of neutralizing antibodies against $G_s\alpha$ causes meiosis to resume, supporting the hypothesis that *X. laevis* $G_s\alpha$ plays a dominant role in maintaining meiotic arrest. In contrast, other groups showed evidence that the Gβγ dimer may be responsible for maintaining *X. laevis* oocyte G_2 arrest (Lutz et al., 2000) or that both $G_s\alpha$ and Gβγ cooperate in activating the cAMP signaling pathway (Guzman et al., 2005; Romo et al., 2002). In either case, these studies suggest the existence of an activated trimeric G protein(s) in oocytes that maintains high levels of cAMP and meiotic arrest, raising the possibility that a membrane bound receptor is required for maintaining the active $G_s\alpha$ and/or Gβγ subunits at high enough concentrations to sustain a meiotic arrest.

Recent studies have identified the orphan G-protein coupled receptor, GPR3, as a G_s activator and an essential mediator of meiotic arrest in mouse oocytes (Hinckley et al., 2005; Mehlmann, 2005; Mehlmann et al., 2004). In addition, the related protein GPR12 has also been proposed to play a critical role in the control of cAMP levels and in meiotic arrest in rat oocytes (Hinckley et al., 2005). GPR3, GPR12, and a third orphan receptor, GPR6, are closely related members of the MECA (melanocortin receptor, EDG receptor, cannabinoid receptor, adenosine-binding receptor) cluster of the α-group of the rhodopsin family of GPCRs (Fredriksson et al., 2003). The roles of GPR3 and GPR12 in mammalian meiotic arrest suggest that some member of the GPR3/6/12 family may mediate meiotic arrest in frog oocytes.

Here we have cloned and characterized a *X. laevis* G-protein coupled receptor related to GPR3, GPR6, and GPR12. Although its closest human relative is GPR12, it is not the *X. laevis* GPR12 homolog, but rather a novel G-protein coupled receptor of a family found in fish and amphibians, but not mammals. We term this protein GPRx and present functional evidence that GPRx is important for maintenance of meiotic arrest in *X. laevis* oocytes.

Materials and methods

Database searches

All homology searches were performed using basic local alignment search tool (BLAST) at the TIGR Unique Gene Indices. The amino acid sequence of the mouse GPR12 (GenBank Accession No. NP20032177), human GPR12 (NP005279), mouse GPR3 (NP032180) and human GPR3 (NP005272) were used to search the *X. laevis* database at TIGR. One of the expressed sequence tags (ESTs) displayed (GenBank Accession No. AW158095) scored the highest sequence identity with the C-terminal end of all four proteins. This sequence was used to design primers for the amplification of *X. laevis* GPR12 relatives.

Cloning and sequencing

The method of rapid amplification of cDNA ends (RACE) (Invitrogen catalog no. 18374-058) was used to amplify the 5′-end of AW158095. Total RNA was isolated from stage VI oocytes using the RNeasy Midi kit (Qiagen catalog no. 75144), and 5′-RACE was performed according to the manufacture's protocol using the primers GSP1 (5′-ATC TGC TAC CAG CGA ATA AAC-3′), GSP2 (5′-AAA GGT GCC CAG TAT AAG GGA-3′) and nested GSP (5′-GTT GAA ACC CCC TTC CTG GTA-3′). The 5′-RACE products were run on a 1% agarose gel containing 0.1 μg/ml ethidium bromide. A single band was purified using the QIAquick Gel Extraction kit (Qiagen catalog no. 28704) and sent for sequencing. Finally, the whole coding region of this clone, termed GPRx, FLAG epitope-tagged at the C-terminus, was amplified from stage VI oocyte double stranded cDNA by ThermoScript RT-PCR System (Invitrogen catalog no. 11146-040), subcloned into pCS107 to allow production of synthetic mRNAs in vitro, and sequenced. The final set of primers used were: 5′-CGC CGC GGA TCC ATG CTT CAC CAG CCT GCA GTC-3′ and 5′-CGG CGG GAATTC ACT CTT GTC GTC ATC GTC CTT GTA GTC TAC GTC ACT GGA AGT TCT-3′. These primers include *Bam*HI and *Eco*RI restrictions sites.

Analysis of protein sequence

Transmembrane segments were identified by the hidden Markov model for topology prediction (HMMTOP) method (Tusnady and Simon, 1998; Tusnady and Simon, 2001). Alignments of protein sequences were performed using Clustal Qt and phylogenetic trees were derived from these alignments using Njplot.

RNA extraction and QPCR

RNA was extracted using RNeasy Maxi kit (Qiagen catalog no. 75162), treated with DNAse I (Sigma catalog no. AMP-D1) and reverse transcribed with the ThermoScript RT-PCR System (Invitrogen catalog no. 11146-040), using 1 μg of RNA from *X. laevis* oocytes, embryos, or tissues. Quantitative RT-PCR reactions were performed in triplicate using one tenth of the cDNA reaction, 100 nM primers, and 2.5 μl of 20× EvaGreen (Biotium, Hayward, CA) in a 50 μl reaction. Primer pairs were as follows: GPRx 5′TCT CTA CAA TGC CCT CAC C-3′ and 5′-TCC CTG AAA CAG TTC CAC C-3′; L32 5′-AAA TTG AAG GAG TCT ATG CTC G-3′ and 5′-TCA CCT TTC CCC AGA TCA C-3′. Reactions were performed on a DNA Engine Opticon 2 Real-Time PCR Detection System using the following cycling conditions: 94 °C 30 s, 55 °C 30 s, 72 °C 45 s for 40 cycles. PCR product specificity was verified with a melting curve and by running on agarose gel electrophoresis. Relative expression analyses were performed using the $\Delta\Delta C_T$ method normalized to *X. laevis* ribosomal protein L32 mRNA. Tissues were normalized to ovary, oocytes and embryos to stage 6 oocytes treated with progesterone.

Oocytes

Pieces of ovary were removed from anesthetized female *X. laevis*. Stage VI oocytes were isolated by treatment with 0.2% collagenase (Sigma catalog no. C-0130) in modified Barth's solution (MBS) without Ca^{2+} (88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 2.5 mM $NaHCO_3$, and 5 mM HEPES pH 7.8) for 1–1.5 h at room temperature. Collagenase-treated oocytes were washed five times with MBS and three times with OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES pH 7.8), and were kept overnight at 16 °C on agarose dishes in a medium composed of 50% Leibovitz's L-15 medium with L-glutamine (GIBCO catalog no. 11417-064)), 15 mM HEPES pH 7.9, 50 μg/ml gentamicin (Sigma catalog no. G1272), and 5% fetal bovine serum (GIBCO catalog no. 26140-079). Defolliculated stage VI oocytes were manually selected, and injections were performed.

Morpholinos and mRNA injections

A morpholino antisense oligo targeted to GPRx was designed by Gene Tools, LLC (Philomath, OR, www.gene-tools.com) based on the cDNA sequence of *X. laevis* GPRx. The morpholino sequence against the GPRx mRNA is 5′-AGGCTGGTGAAGCATTGCTA-TATGC-3′. The standard control morpholino (5′-CCTCTTACCTCAgTTACAATTTATA-3′) was also purchased from Gene Tools, LLC. All morpholinos were dissolved in RNase-free water at 1.0 mM stock concentration. GPRx mRNA was generated through in vitro transcription using linearized plasmid DNA as template and the mMESSAGE mMACHINE kit (Ambion catalog no. 1340) with SP6 polymerase. GPRx mRNA was diluted to 1 μg/μl stock concentration. Subsequent injections were carried out in OR2 solution using a nanoinjector (Drummond Scientific). Rescue experiments were performed by co-injecting the morpholino with the mRNA. After 24–36 h, maturation was induced with 1.5 μM progesterone in OR2 solution and GVBD was scored by the appearance of a white spot in the animal pole of the oocyte.

Oocyte lysis

Oocytes were lysed by pipetting up and down in 10 μl/oocyte lysis buffer (0.25 M sucrose, 100 mM NaCl, 2.5 mM $MgCl_2$, 20 mM HEPES, pH 7.2) containing 10 mM EDTA, protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 mM PMSF), and phosphatase inhibitors (50 mM 2-glycerophosphate, 1 mM Na_3VO_4, 2 mM NaF). Samples were clarified by centrifugation for 5 min in a Beckman E microcentrifuge (Fullerton, CA) with a right-angle rotor. The clarified supernatant was removed and mixed with an equal volume of 2× Laemmli sample buffer for subsequent SDS-PAGE.

Oocyte membrane preparation

Oocytes (typically 30–50) were homogenized with a Dounce homogenizer (pestle A, 15 strokes) in 10 μl/oocyte membrane buffer (83 mM NaCl, 1 mM $MgCl_2$, 10 mM Hepes

pH 7.6, 10 mM NaF) containing phosphatase inhibitors (50 mM 2-glycerophosphate, and 1 mM Na_3VO_4), and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 10 μg/ml pepstatin, and 1 mM PMSF) at 4 °C. The homogenate was centrifuged at 1000×g for 10 min, and the supernatant was removed and centrifuged again at 1000×g. This supernatant was then centrifuged using a Beckman/Coulter TLA-100.3 rotor and Beckman polyallomer microfuge tubes at 95,000 rpm for 30 min or at 14,000 rpm for 60 min using a Tomy MTX-150 refrigerated microcentrifuge in standard microcentrifuge tubes. The supernatant was saved and the membrane pellet was resuspended in membrane buffer (10 μl/oocyte). The suspension was centrifuged one more time at 95,000 rpm for 30 min or 14,000 rpm for 60 min, and this final pellet was resuspended in buffer (50 mM Tris pH 6.8, and 4% SDS). Protein concentration was determined by the Bradford method and samples were stored at −80 °C until needed.

Antibody production

Peptide antibodies directed against the N-terminus of GPRx were raised in rabbits using the following peptide conjugated to keyhole limpet hemocyanin and ovalbumin for immunization: Ac-HLHNDSSGRIFHHQHDPWC-amide. Peptide synthesis, immunizations, characterization with ELISA method, and affinity purification of the antibodies were performed by Quality Controlled Biochemicals (Hopkinton, MA).

Western blotting

Samples of oocyte extracts and membrane fractions were run on SDS-PAGE gels and transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore catalog no. IPVH00010), which were then blocked with 3% non-fat milk in Tris-buffered saline (150 mM NaCl and 20 mM Tris; pH 7.6) and incubated for 1 h with a 1:1000 dilution of one of the following primary antibodies: anti-MAPK (DC3, raised in our laboratory), anti-GPRx, or anti-FLAG M2 (SIGMA catalog no. F3165). Blots were washed three times with Tris-buffered saline plus 0.1% Tween-20 and probed with horseradish peroxidase (HRP)-conjugated secondary antibody (Zymed catalog no. 81-6120 or 81-6720) for 1 h. The signal was detected by Immun-Star-HRP Chemiluminescent system (Bio-Rad catalog no. 170-5041).

Deglycosylation

Deglycosylation of GPRx with endoglycosidase H (Endo H) (New England BioLabs catalog no. P0702L) and/or *N*-glycosidase F (PNGase F) (New England BioLabs catalog no. P0704S) was carried out according to the manufacturer's protocol. The products of the reaction were separated by SDS-PAGE and western blotting with anti-GPRx.

In vitro translation

In vitro translation from GPRx mRNA, or coupled transcription/translation from an SP6 promoter and GPRx cDNA, were carried out with a TnT rabbit reticulocyte lysate kit (Promega catalog no. L4600). Alternatively, we used *X. laevis* egg extracts (Smythe and Newport, 1991) for the in vitro translation of GPRx, as described (Matthews and Colman, 1991).

cAMP measurements

We determined cAMP by using Assay Designs' cyclic AMP Complete Enzyme Immunometric Assay kit (Assay Designs catalog no. 900-163). We lysed 10 oocytes, by forcing them through pipette tips, in 250 μl of ice-cold 95% ethanol. Extracts were centrifuged at 15,000×g for 15 min at 4 °C. The supernatants were transferred to new tubes and dried under vacuum. The residue was dissolved, subjected to acetylation, and cAMP was measured according to the kit's provided protocol.

Results

Cloning and sequence analysis of X. laevis GPRx

To find a *X. laevis* homolog of mammalian GPR3 or GPR12 we searched the *X. laevis* database at TIGR Unique Gene Indices and identified the EST AW158095 as the best candidate for *X. laevis* GPR12. The EST was amplified from a *X. laevis* cDNA pool by 5′ RACE (see Materials and methods) and was subcloned into pCS107. The cDNA clone obtained was 1434 nucleotides (nt) in length, including a 5′ untranslated region (UTR) of 331 nt and 3′-UTR of 63 nt excluding the poly(A) tail (accession number 895822). The ORF encodes a predicted protein of 340 residues with a molecular weight of 37,477. There are three in frame stop codons upstream of this ORF. The predicted protein product contains the seven transmembrane (TM) regions characteristic of G-protein coupled receptors (Fig. 1A) (Tusnady and Simon, 1998; Tusnady and Simon, 2001). There is no obvious signal peptide or signal peptidase cleavage site at the N-terminus of the protein; as is the case with other GPCRs, the first transmembrane sequence may perform this function.

The closest mouse and human relatives of our predicted protein were GPR12 proteins, followed by GPR3 and GPR6. Overall, the amino acid identity was 48% with mouse GPR6, 51% with mouse GPR3, and 63% with mouse GPR12.

We found three *Xenopus tropicalis* clones close in sequence to our protein (JGI accession numbers 305630, 404365, and 418969), and examined the relationships between these proteins, our protein, and various other vertebrate GPR3/6/12 proteins. We carried out a multiple sequence alignment of all of the vertebrate GPR3/6/12-like proteins and constructed a bootstrapped neighbor-joining radial tree (using Clustal Qt). As shown in Table 1 and Fig. 1B, one *X. tropicalis* sequence was the clear homolog of our protein. The other two represent the *X. tropicalis* homologs of mouse GPR6 and mouse GPR12 (Fig. 1B). This indicated that our protein was not the *X. laevis* GPR12 ortholog, but rather a member of a distinct group of GPCRs. Consistent with this identification, the zebrafish genome includes both a GPR12 homolog and two GPCRs more closely related to our protein (Fig. 1B). We therefore suggest the name GPRx for this new subgroup of GPR3/6/12-related proteins (Fig. 1B) found, so far, only in amphibians and fish.

Tissue distribution of GPRx RNA

To assess the tissue distribution of GPRx we performed quantitative RT-PCR on RNA extracted from various tissues. As observed in Fig. 2A, GPRx is expressed predominantly in brain, with ovary and testis being the next richest sources. Since we originally cloned GPRx from stage VI oocyte cDNA, we wanted to assess if GPRx expression was regulated throughout oogenesis. We treated ovaries with collagenase, manually sorted oocytes from stages I to VI, and performed qRT-PCR, using the ribosomal protein L32 for normalization (Pierandrei-Amaldi et al., 1982). As observed in Fig. 2B, GPRx RNA expression was detectable in stage I oocytes and the amount of GPRx RNA increased several fold during oogenesis. We also fertilized eggs and assessed GPRx RNA levels (normalized per embryo) at various stages of embryonic development. The GPRx RNA persisted until after the midblastula transition (Fig. 2C). Thus GPRx RNA is expressed at the right time and in the right tissues to make it a possible regulator of oocyte maturation.

Characterization of the GPRx protein

To begin to characterize the GPRx protein, we translated a C-terminal Flag-tagged GPRx mRNA in reticulocyte lysates. As shown in Fig. 3A, the main ^{35}S-labeled band migrated with an apparent molecular mass of 28 kDa, smaller than the predicted size of the GPRx protein (37 kDa). This band was recognized by affinity-purified antibodies raised against an N-terminal GPRx peptide (Fig. 3A) and also by Flag antibodies (not shown), indicating that despite its small apparent molecular mass, it represented the full-length GPRx protein. As described below, the 28 kDa band did not shift or disappear after treatment with endoglycosidase H or *N*-glycosidase F (Fig. 3B and data not shown). Thus we interpret the p28 GPRx band as representing the non-glycosylated or minimally glycosylated primary translation product of GPRx.

However, when microinjected into *X. laevis* oocytes, the same GPRx mRNA yielded two major immunoreactive bands at 28 and 35 kDa (Fig. 3B). Similar results were obtained when the GPRx mRNA was translated in *X. laevis* egg extracts (Fig. 3C). Our working hypothesis was that the 35 kDa band represented glycosylated GPRx. Both bands could be detected in a 95,000 rpm pellet (Fig. 3D) that contained plasma membrane (Na/K ATPase) and endoplasmic reticulum (calnexin) markers (not shown). However in some membrane preparations (not shown) the 28 kDa GPRx band was underrepresented relative to the 35 kDa band, consistent with the

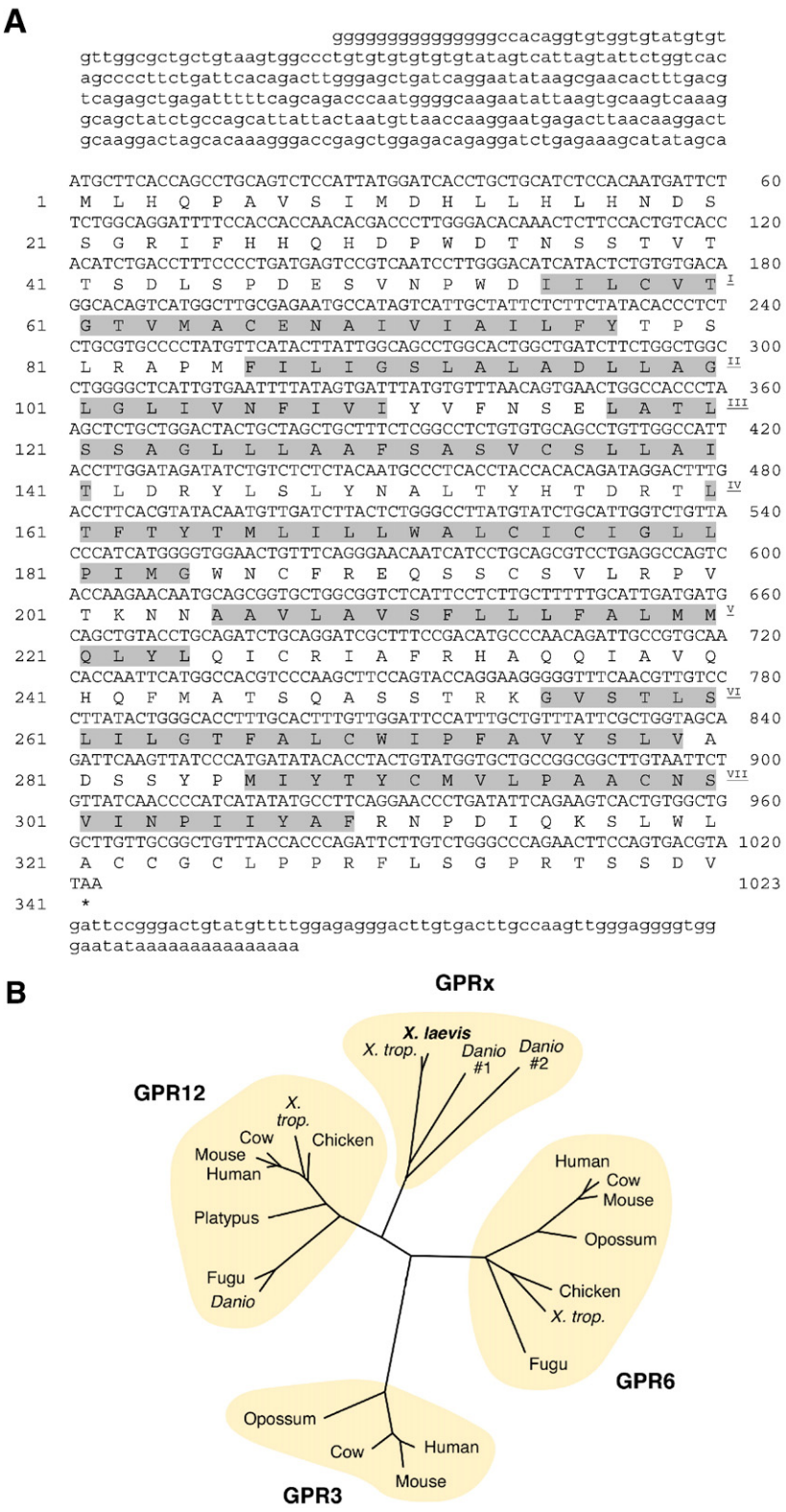

Fig. 1. GPRx and its relationship to GPR3, GPR6, and GPR12. (A) The nucleotide and deduced amino acid sequence of *Xenopus laevis* GPRx. The GenBank accession number is 895822. Nucleotides are numbered to the right and amino acids to the left. Lowercase letters indicate untranslated regions. The predicted transmembrane domains are shaded and are numbered with Roman numerals. (B) Radial tree obtained using ClustalQt and Njplot showing the relationships between the GPR3, GPR6, GPR12, and GPRx proteins. This unrooted neighbor-joining tree was constructed by bootstrapping using 1000 seeds. Because one sequence (chicken GPR6) contained a long N-terminal extension absent from the other GPR sequences, positions with gaps were ignored in constructing the tree.

possibility that only a fraction of the 28 kDa protein was membrane-associated. Treatment of the membrane pellet with endoglycosidase H caused the 35 kDa band to disappear and the 28 kDa band to increase in intensity (Fig. 3B, lane 2), without affecting the intensities of two higher molecular weight (50 and 60 kDa) bands. Similar results were obtained with *N*-glycosidase F (not shown). Thus the 35 kDa band

Table 1
G-protein coupled receptors related to GPRx

Protein	Species	Source	Accession number	NCBI/JGI definition
GPR3	Human	NCBI	NP_005272	G protein-coupled receptor 3
	Mouse	NCBI	NP_032180	G protein-coupled receptor 3
	Cow	NCBI	XP_612644	Similar to G protein-coupled receptor GPR3
	Opossum	NCBI	XP_001371085	Similar to G protein-coupled receptor GPR3
GPR6	Human	NCBI	NP_005275	G protein-coupled receptor 6
	Mouse	NCBI	AAI11862	G protein-coupled receptor 6
	Cow	NCBI	NP_001093777	Hypothetical protein LOC50666
	Opossum	NCBI	XP_001368592	Similar to putative G protein-coupled receptor
	Chicken	NCBI	XP_426182	Hypothetical protein
	Fugu	JGI	572864	–
	X. tropicalis	JGI	418969	Putative ortholog of probable G-protein coupled receptor GPR6
GPR12	Human	NCBI	AAH67449	GPR12 protein
	Mouse	NCBI	XP_601035	G-protein coupled receptor 12
	Cow	NCBI	XP_601035	Similar to putative GPR12 G protein coupled-receptor
	Opossum	NCBI	XP_001366959	Similar to putative GPR12 G protein coupled-receptor
	Platypus	NCBI	XP_001519402	Similar to GPR12 protein
	Chicken	NCBI	XP_425637	Similar to GPR12 protein
	X. tropicalis	JGI	404365	Putative ortholog of probable G-protein coupled receptor GPR12
	Danio rerio	NCBI	XP_693970	Hypothetical protein
	Fugu	JGI	588755	–
GPRx	*X. laevis*	NCBI	NP_001104190	GPRx
	X. tropicalis	JGI	305630	–
GPRx-1	*Danio rerio*	NCBI	XP_693580	Hypothetical protein
GPRx-2	*Danio rerio*	NCBI	XP_001339092	Hypothetical protein

seen in GPRx-overexpressing *X. laevis* egg extracts and oocytes appears to be a more highly glycosylated form of the 28 kDa band.

A faint endogenous 28 kDa GPRx band was also detected in oocyte membranes (Fig. 3B, lanes 3 and 4; Fig. 3D, left; Fig. 4A). However, the 35 kDa GPRx band was not detected (Figs. 3B, D), raising the possibility that it is either extremely scarce or it is processed further in oocytes to a form with a different molecular weight. Consistent with the latter possibility, two other bands were seen in GPRx immunoblots of oocyte membranes, at 50 kDa and 60 kDa (Figs. 3B, D). Both the p50 and p60 bands were present in 95,000 rpm membrane pellets, whereas only the p50 band was detected in lower speed pellets (Fig. 3D, right, and E). To further test the identities of the 50 and 60 kDa bands, we compared immunoblots from control oocytes and oocytes micro-injected with a GPRx morpholino oligonucleotide. The 28 and 50 kDa bands were greatly diminished in oocytes injected with a GPRx morpholino oligonucleotide (Fig. 3D, left; E; see also Fig. 4A), but the 60 kDa was unaffected. A number of bands were seen in the supernatant, but none disappeared from the morpholino-injected sample (Fig. 3D). We repeated the analysis using a lower speed (14,000×*g*) centrifugation and found one main band in the pellet, the 50 kDa band, which, again, was decreased in intensity in oocytes injection with GPRx morpholinos (Fig. 3E). This indicates that p28 GPRx and the p50 band are likely to be GPRx-related, whereas the p60 band is likely to be a cross-reacting protein. As shown in Fig. 3E, p50 levels increased during oogenesis, plateauing around stages III–IV, and remained roughly constant during oocyte maturation.

Other members of the GPR3/6/12 family have been shown to increase intracellular cAMP concentrations (Freudzon et al., 2005; Hinckley et al., 2005; Mehlmann et al., 2004; Norris et al., 2007; Tanaka et al., 2007; Uhlenbrock et al., 2002). We were therefore interested in determining if the same was true of GPRx. As shown in Fig. 3F, the expression of GPRx in oocytes increased cAMP levels by approximately 60%. Under these conditions the receptor is estimated to be overexpressed ~2–4-fold (e.g. Fig. 3B), consistent with the hypothesis that GPRx is not the only GPCR contributing to the oocyte's cAMP levels. Under conditions where GPRx morpholinos decreased protein levels substantially (Figs. 3D, E), there was no measurable change in cAMP levels (<9%; Fig. 3F), again consistent with the hypothesis that GPRx is not solely responsible for cAMP production.

Morpholino injections accelerate progesterone-induced oocyte maturation

Although we saw no obvious decrease in cAMP levels in GPRx-morpholino-injected oocytes, the magnitude of cAMP decrease

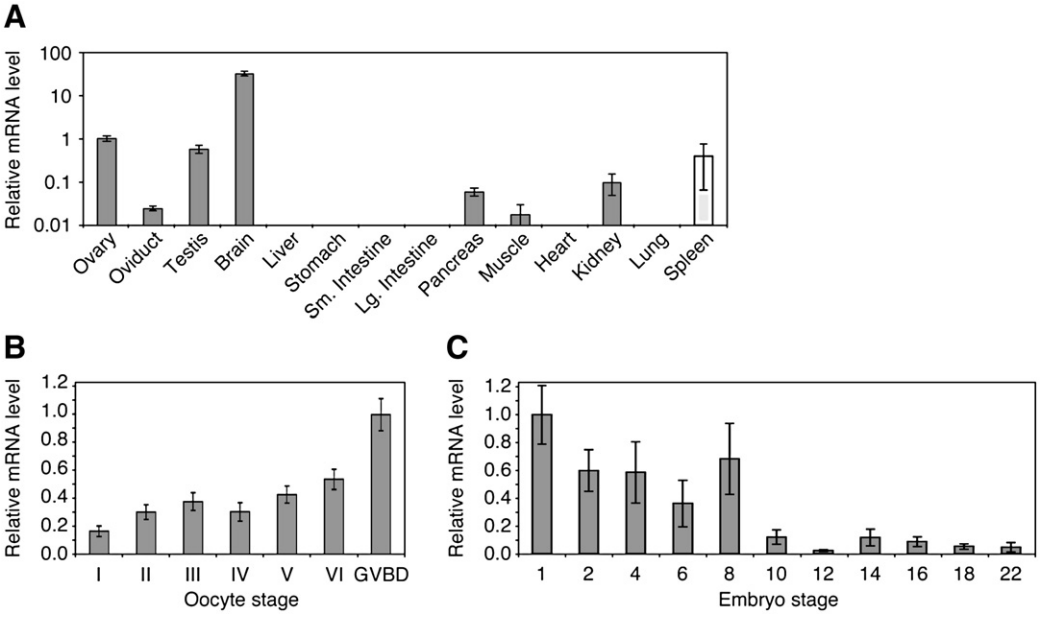

Fig. 2. GPRx RNA expression. (A) Quantitative RT-PCR analysis of GPRx expression in *Xenopus laevis* tissues. The GPRx levels were normalized to ribosomal protein L32, with both transcripts quantified by RT-PCR. (B) Quantitative RT-PCR analysis of GPRx expression through oogenesis. As in panel A, the GPRx was normalized to *Xenopus laevis* ribosomal protein L32 mRNA. (C) Quantitative RT-PCR analysis of GPRx expression during embryogenesis. GPRx was normalized per embryo.

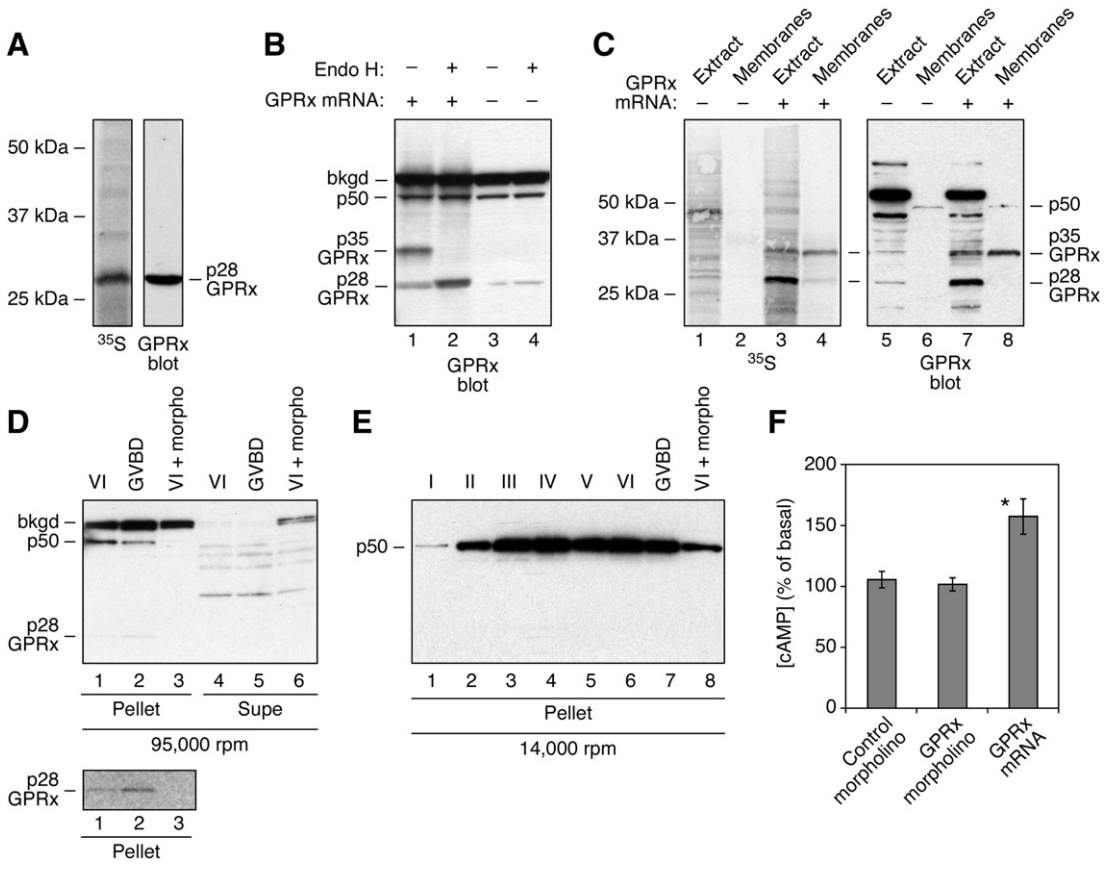

Fig. 3. Characterization of the GPRx protein. (A) In vitro transcription and translation of GPRx in rabbit reticulocyte lysates. The GPRx cDNA (1 μg per reaction) was transcribed with SP6-RNA polymerase and translated in the presence of [^{35}S]methionine. The reactions were analyzed by SDS PAGE followed by autoradiography and GPRx immunoblotting. (B) Ectopic expression of GPRx in *Xenopus laevis* oocytes. Oocytes were injected with GPRx mRNA (32 ng/oocyte) and allowed to express for 24 h. Membranes were prepared by centrifugation at 12,000 rpm and treated with or without endoglycosidase H (Endo H, 500 U per reaction) for 1 h. Proteins were analyzed by GPRx immunoblotting. (C) In vitro translation of GPRx in *Xenopus laevis* egg extracts. GPRx mRNA (1 μg) was translated in the presence of [^{35}S]methionine. Samples of the whole reaction mixture ("Extract") or membrane fractions ("Membrane") were analyzed by autoradiography and GPRx immunoblotting. (D) Endogenous oocyte proteins recognized by the GPRx antibody. Membranes were prepared by 95,000 rpm centrifugation. Lane 1, membranes from stage VI oocytes; lane 2, membranes from stage VI oocytes treated with 5 μM progesterone to induce maturation; lane 3, membranes from stage VI oocytes injected with 64 ng morpholinos; lane 4, supernatant from stage VI oocytes; lane 5, supernatant from stage VI oocytes treated with 5 μM progesterone to induce maturation; lane 6, supernatant from stage VI oocytes injected with 64 ng morpholinos. The inset shows a section of the same blot with the contrast and brightness adjusted to make the faint p28 band more apparent. (E) Accumulation of the p50 protein during oogenesis. Membranes were prepared by 14,000 rpm centrifugation. From lane 1 to 6, all stages oocytes; lane 7, membranes from stage VI oocytes treated with 5 μM progesterone to induce maturation; lane 8, membranes from stage VI oocytes injected with 64 ng morpholinos. (F) cAMP levels in oocytes microinjected with GPRx mRNA (23 ng/oocyte), GPRx morpholino oligonucleotides 51 ng/oocyte), or control morpholinos (50 ng/oocyte). Data are shown as means ± S.E. from 20 experiments and are expressed relative to uninjected controls. The difference between the cAMP levels in the GPRx mRNA-injected oocytes and uninjected controls was statistically significant by the Student's *t*-test (P=0.0009). The difference between the cAMP levels in the GPRx morpholino-injected oocytes and the control morpholino-injected oocytes was 3 ± 8.6%, which was not significant.

required to induce oocyte maturation is modest (Maller et al., 1979a; Schorderet-Slatkine et al., 1982), raising the possibility that the contribution of GPRx towards maintaining cAMP levels may still be functionally significant. To explore this further, we examined the kinetics of oocyte maturation in GPRx-morpholino-injected oocytes. The morpholinos were again effective in decreasing the intensity of both the p50 band and p28 GPRx (Fig. 4A, with membranes prepared at 95,000×*g* to allow detection of both bands), whereas control morpholinos had little or no effect (Fig. 4A). Morpholino-injected oocytes did not spontaneously mature, even 6 days post-injection (not shown), but progesterone-treated morpholino-injected oocytes underwent GVBD approximately 30 min earlier than progesterone-treated uninjected and control-injected oocytes did (Fig. 4B). The morpholino-injected oocytes also showed earlier activation of p42 MAPK, a biochemical correlate of GVBD, compared to uninjected and control-injected oocytes (Fig. 4C). These results demonstrate that the reduction of GPRx accelerates progesterone-induced oocyte activation, and suggest that GPRx is a negative regulator of oocyte maturation, either through its contribution to maintaining cAMP or through some other as yet unidentified signaling pathway.

Injection of GPRx mRNA blocks progesterone-induced oocyte activation

To determine whether overexpression of p28 and p35 GPRx can disrupt maturation, we injected oocytes with different concentrations of GPRx mRNA and scored for GVBD after 8 h of progesterone treatment. We found that oocytes injected with concentrations of mRNA higher than 0.5 ng failed to undergo GVBD and activation of MAPK (Fig. 5A). mRNA concentrations lower than 0.5 ng of mRNA blocked maturation in a fraction of the oocytes (Fig. 5A). This is consistent with the hypothesis that GPRx normally functions as an inhibitor of maturation.

To test the specificity of this overexpression effect, we co-injected 70 ng of morpholinos with different concentrations of GPRx mRNA. As shown in Fig. 5B, the GPRx morpholinos blocked the effect of GPRx mRNA on maturation and p42 MAPK activation. This rescue shows that the suppression of maturation is not due to general toxicity of the GPRx mRNA, but rather depends upon the production of GPRx protein. The rescue also serves to corroborate the specificity of the morpholino effect, since the morpholino's ability to accelerate progesterone-induced maturation

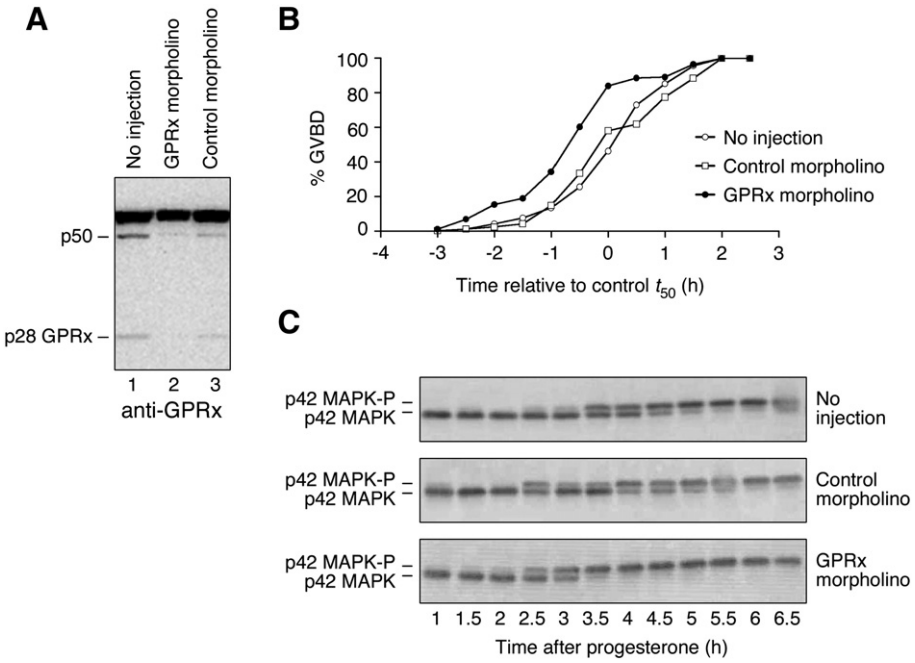

Fig. 4. Effects of GPRx morpholino oligonucleotides on progesterone-induced maturation. (A) Immunoblot of *Xenopus laevis* oocyte membranes showing knock-down of endogenous GPRx. Total protein is 20 μg/lane. (B) Modest acceleration of GVBD in response to injection of morpholinos against GPRx into *Xenopus laevis* oocytes. Oocytes were injected with 50 to 130 ng of control morpholinos or GPRx morpholinos, incubated 24 h, and scored for GVBD at various time after 1.5 μM progesterone stimulation. Data are taken from five separate experiments. For each experiment we defined $t=0$ as the time when 50% of the control oocytes had matured. (C) Modest acceleration of p42 MAPK activation in morpholino-injected oocytes. The data shown are from one of the five experiments included in panel B, with 10 randomly-selected oocytes taken per time point.

(Fig. 4B) is reversed by the GRPx mRNA in a dose-dependent fashion (Fig. 5B).

Discussion

Here we report the cloning and characterization of GPRx, a new member of the GPR3/6/12 family of G-protein coupled receptors. The closest human homolog of GPRx is GPR12. However, GPRx is not the *X. laevis* ortholog of GPR12, but rather a member of a separate branch of the GPR3/6/12-family tree (Fig. 1). GPRx orthologs are present in *X. laevis*, *X. tropicalis*, and *Danio rerio* (zebrafish). However no GPRx homologs have been cloned from chicken, mice, or humans; so far the GPRx subgroup of GPR3/6/12 receptors has only been found in amphibians and fish. GPRx is expressed at highest levels in brain, but is also expressed at relatively high levels in ovary and testis (Fig. 2A). The GPRx RNA increases in abundance during oogenesis, plateauing at around stage IV, and is maintained until after the midblastula transition.

We have detected at least two and possibly three forms of the GPRx protein. The first is p28 GPRx. This is the main form produced by in vitro translation in reticulocyte lysates (Fig. 3A) and *X. laevis* egg lysates (Fig. 3C), and is detected in *X. laevis* oocytes as well (Figs. 3B, D and 4A). p28 GPRx probably represents the full-length, hypoglycosylated or non-glycosylated form of the protein. The second form of GPRx is p35 GPRx, which is detected after in vitro translation of GPRx in *X. laevis* egg lysates or expression of GPRx in intact oocytes (Figs. 3B, C). p35 GPRx represents a more heavily glycosylated form of p28 GPRx (Fig. 3B). The third possible form of GPRx is p50, a protein present in *X. laevis* membrane preparations that is recognized by affinity-purified GPRx antibodies and knocked down by GPRx morpholinos (Figs. 3B, D, E and 4A). One possibility is that p50 represents the most fully glycosylated form of GPRx, and that it is difficult to get this full processing in either *X. laevis* extracts or in oocytes during overexpression. However, neither endoglycosidase H (Fig. 3B, lanes 3 and 4) nor *N*-glycosidase F (not shown) caused the p50 band to disappear or shift to a lower molecular weight, indicating that p50 either is not heavily glycosylated or is glycosidase-resistant. A second possibility is that p50 represents a GPRx dimer. However, variations in the buffer used for preparing gel samples (e.g. 2.3% to 10% SDS; boiled vs. 37 °C vs. 4 °C samples) did not affect the yield of p50 (data not shown). Another possibility is that p50 is GPRx translated from an alternatively spliced transcript, or that the pseudo-tetraploid *X. laevis* possesses both a long and a short GPRx gene. Consistent with either of these possibilities, the cDNA reported for chicken GPR6 encodes a protein that is about twice as big (675 aa) as the other known vertebrate GPR3/6/12/x family members (330–340 aa). Finally, it is possible that p50 is only distantly related to GPRx and that its knockdown with GPRx morpholinos was an off-target effect.

The expression of GPRx in fully-grown oocytes suggests some role in oocyte maturation. Its relatives GPR12 and GPR3 have been shown to contribute to the maintenance of meiotic arrest in mouse and rat oocytes (Freudzon et al., 2005; Hinckley et al., 2005; Mehlmann, 2005; Mehlmann et al., 2004), and our studies indicate a similar role for GPRx in *X. laevis* oocytes. Like GPR3/6/12, GPRx expression increases cAMP levels (Fig. 3F). Moreover, knocking-down GPRx expression accelerates progesterone-induced maturation, and overexpression of GPRx inhibits progesterone-induced maturation (Figs. 4 and 5). One caveat to this finding is that when GPRx is overexpressed in oocytes, a 35 kDa form that is not detectable in non-injected oocytes is produced (Fig. 3B). This p35 GPRx may represent a partially glycosylated form, with p50 representing the natural, fully processed GPRx protein, or it may represent a fully glycosylated form that is simply too scarce to detect in uninjected oocytes. Note, however, that the exact glycosylation state of GPRx may not be critical for the tonic activation of adenylyl cyclase.

Our current view of the initial events in progesterone-induced oocyte maturation is shown in Fig. 6. GPRx helps maintain adenylate cyclase activation and opposes oocyte maturation. Some other progesterone-responsive protein, with XmPRβ being the most plausible candidate, then triggers the change in balance between cAMP synthesis and destruction that brings about oocyte maturation.

Several important questions remain unanswered. It seems likely that other GPCRs contribute to G2 arrest in *X. laevis* oocytes, but it is

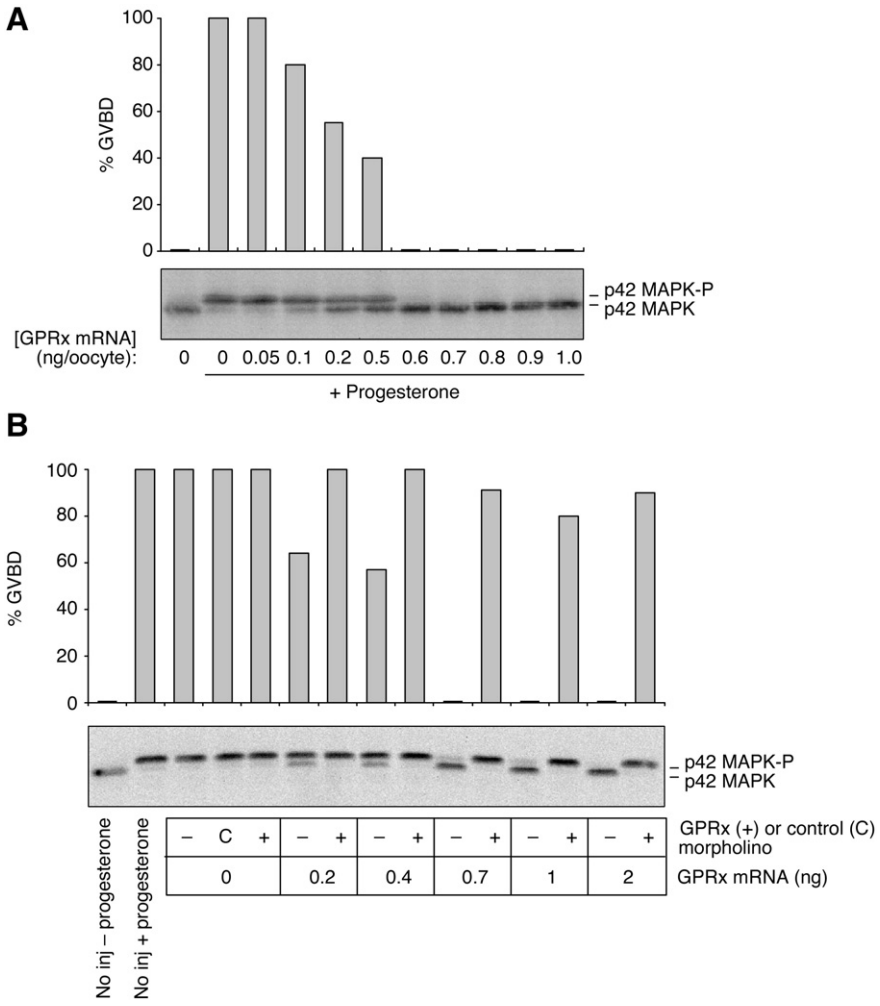

Fig. 5. Overexpression of GPRx inhibits maturation and GPRx morpholinos rescue maturation. (A) Overexpression of GPRx inhibits progesterone-induced maturation. Oocytes were injected with different concentrations of GPRx mRNA, incubated 36 h, and then scored for GVBD after 8 h of treatment with 1.5 μM progesterone. Each bar/lane represents data from 25 oocytes. Results are representative of three independent experiments. (B) Morpholinos rescue progesterone induced maturation in oocytes injected with GPRx mRNA. Oocytes were injected with different concentrations of GPRx mRNA with or without GPRx morpholinos (70 ng per oocyte), incubated 24 h, and scored for GVBD after 8 h of 1.5 μM progesterone stimulation. Oocytes were then lysed and subjected to MAPK immunoblotting. Each bar/lane represents data from 25 oocytes.

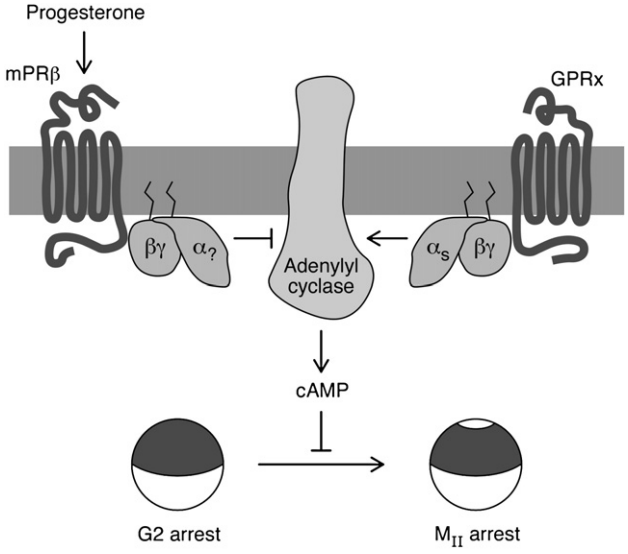

Fig. 6. Schematic view of the role of GPRx in maintaining meiotic arrest.

not clear what those receptors are. Given that GPR3 orthologs have not been detected outside of mammals (Fig. 1B), we suspect the relevant protein(s) may be GPR6 and/or GPR12. However, the *X. laevis* GPR6 and GPR12 genes have not yet been identified, although sequences for both are available from the closely related species *X. tropicalis* (Table 1). Oocytes also express numerous G-protein coupled receptors outside of the GPR3/6/12/x subfamily (Hinckley et al., 2005). The roles of these receptors in oocyte physiology are largely unknown; it seems plausible that some contribute to the regulation of cAMP levels and the establishment of the G2 arrest state.

In addition, it is not know whether GPRx's activity is modulated. One possibility is that GPRx may be regulated by progesterone or androgens, as is true of XmPRβ, by ligands like sphingosine-1-phosphate that have been implicated as potential agonists at other GPR3/6/12 family receptors, or by other unidentified ligands. The answers to these questions may help us to better understand the non-genomic effects of steroid hormones and the biochemical physiology of oocyte maturation.

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