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MOLECULAR CLONING AND HORMONAL CONTROL IN THE OVARY OF CONNEXIN 31.5 mRNA AND CORRELATION WITH THE APPEARANCE OF OOCYTE MATURATIONAL COMPETENCE IN RED SEABREAM

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Summary

Gap junctions are aggregates of intercellular channels, composed of the protein connexin (Cx), between adjacent cells. This study examined whether, in the ovary of the red seabream *Pagrus major*, the connexin gene essential for the production of RNA and protein during the acquisition of oocyte maturational competence is active. Mixed primers for this reaction were designed on the basis of the high sequence homology of selected regions of known connexin genes. Polymerase-chain-reaction-amplified cDNA fragments generated by 3′ and 5′ rapid amplication of cDNA ends were combined to generate full-length cDNA sequences. The resulting 2400 base pair cDNA had an open reading frame encoding a polypeptide containing 275 amino acid residues (31493 Da; Cx31.5). Hydropathicity analysis of the predicted amino acid sequence indicated that red seabream Cx31.5 has four major hydrophobic regions and four major hydrophilic regions indicative of a topology similar to that of known connexins. Typical connexin consensus sequences were also observed in the first and second extracellular loops. During the acquisition of oocyte maturational competence, red seabream Cx31.5 mRNA transcription levels increased after treatment with gonadotropin-II. It is therefore proposed that expression of Cx31.5 contributes to the acquisition of oocyte maturational competence in this species.

Key words: *Pagrus major*, red seabream, oocyte, maturational competence, connexin, Cx31.5, gonadotropin, germinal vesicle breakdown, RACE.

Introduction

Gap junctions are specialized regions of the plasma membrane of animal cells characterized by intercellular cytoplasmic communication channels that allow direct cell-to-cell passage of ions, metabolites, secondary messengers and small molecules up to 1000 Da in mass, thereby providing a pathway for both electrical and chemical communication between networks of coupled cells (Stagg and Fletcher, 1990). Moreover, gap junctions participate in the regulation of diverse functions, including the contraction of cardiac and smooth muscle, the transmission of neuronal signals at electrotonic synapses, and metabolic cooperation during the development of avascular organs (Kumar and Gilula, 1986; Bruzzone et al., 1996; Grazul-Bilska et al., 1997). The homologous protein found in gap junctions is called connexon, and each connexon molecule is made up of six connexin (Cx) subunits (Beyer et al., 1990; Bruzzone et al., 1996). The predicted molecular mass is used in combination with the generic term to distinguish members of the connexin family. Thus, the 27 kDa protein from rat liver is termed rat Cx27 (Hertzberg et al., 1985; Beyer et al., 1987).

The significance of gap junctions between the oocyte and adjacent granulosa cells has been investigated in the ovarian follicles of several species of fish (Spray et al., 1981; Teranishi et al., 1983). After injecting the fluorescent dye Lucifer Yellow into an oocyte, the dye is normally transferred to the overlying granulosa cells (Cerda et al., 1993). In teleost fish, heterocellular gap junctions were first recognized by electron microscopy in cortical alveolar follicles just prior to the onset of vitellogenesis (Iwamatsu and Ohta, 1981; Kessel et al., 1985). The ovarian follicles of the Atlantic croaker (*Micropogonias undulatus*) contain many heterocellular and homocellular associations and gap junctional contacts during vitellogenesis. Few of these are present after the completion of vitellogenesis, but they can be induced again following the stimulation of maturational competence by human chorionic gonadotropin (hCG) (York et al., 1993). This indicates that gap junction communication plays an important role both during vitellogenesis and in the acquisition of maturational competence.

Yoshizaki et al. (1994) isolated and characterized two ovarian connexin cDNAs from the Atlantic croaker, Cx32.2 and Cx32.7. The increased production of connexin mRNA

appeared to be selective because the level of Cx32.2 increased with gonadotropin (GtH) induction of maturational competence. Cx32.2 is considered essential in the acquisition of oocyte maturational competence (Yoshizaki et al., 1994). However, gonadotropin control of connexin expression in relation to maturational competence has not been examined in any other vertebrate species (Yoshizaki et al., 1994).

Tanaka et al. (1993) recently purified two distinct gonadotropins from the pituitary gland of the red seabream (*Pagrus major*), designated PmGtH-I and PmGtH-II. Both stimulated the *in vitro* production of oestradiol-17β in the vitellogenic follicles of the red seabream, although the biological activity of PmGtH-I was much lower than that of PmGtH-II (Tanaka et al., 1993). Moreover, it is known that priming with an injection of gonadotropin influences the induction of oocyte maturation *in vitro* (Patino and Thomas, 1990; Degani and Boker, 1992). In the oocytes of Atlantic croaker (Patino and Thomas, 1990) and red seabream (Kagawa et al., 1994), actinomycin D, an inhibitor of RNA and protein synthesis, has been found to inhibit gonadotropin-induced germinal vesicle breakdown. These reports suggest that the production of new proteins through RNA synthesis induced by gonadotropin is essential for the development of maturational competence in oocytes (Patino and Thomas, 1990; Kagawa et al., 1994). Except for Atlantic croaker connexin 32.2 mRNA (Yoshizaki et al., 1994), however, gene activity related to oocyte differentiation or maturational competence has not been directly investigated in red seabream or in any other vertebrate species.

The present study was designed to measure changes in the mRNA in the ovarian follicles of red seabream induced by treatment with hCG during the acquisition of oocyte maturational competence. The objectives of the study were to clarify whether 17α,20β-dihydroxy-4-pregnen-3-one (DHP; Sigma, St Louis, MO, USA), human insulin-like growth factor-I (IGF-I; human recombinant, Bachem Inc., Torrance, CA, USA), PmGtH-I or PmGtH-II can induce the acquisition of oocyte maturational competence at the molecular level during the final stage of maturation in the red seabream and to generalize the assumption that the synthesis of mRNA for connexin is essential to this process.

Materials and methods

Fish and tissue collection

Mature red seabream (*Pagrus major*), weighing 2.0–3.0 kg, were purchased from fishermen in Gokasho Bay, Mie Prefecture, Japan, and kept in a flow-through outdoor tank (3000 l) under natural conditions of temperature and photoperiod. Fish were anaesthetized with 2-phenoxyethanol (Sigma) and killed by decapitation at 08:00 h (for incompetent ovaries) and 16:00 h (for competent ovaries). Incompetent ovaries were removed and place in ice-cold Ringer's solution (pH 7.4, supplemented with 10 g l^{-1} NaCl, 0.22 g l^{-1} KCl, 0.25 g l^{-1} $CaCl_2$, 0.74 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1.19 g l^{-1} Hepes, 0.9 g l^{-1} glucose, 0.1 g l^{-1} streptomycin and 100 000 i.u. l^{-1} penicillin G calcium). The ovaries were cut into small pieces in ice-cold Ringer's solution, and ovarian follicles were dispersed by pipetting the fragments.

Incubation procedures

Clusters of ovarian follicles were removed by passing the suspension through a stainless-steel wire mesh (pore diameter approximately 600 μm). Ovarian follicles at the tertiary yolk stage (approximately 500 μm in diameter; Matsuyama et al., 1988) were collected on a wire mesh (pore diameter approximately 420 μm). Approximately 60 ovarian follicles were transferred into individual wells of 24-well culture plates, each of which contained 1 ml of Leibovitz's L-15 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 2.4 g l^{-1} Hepes, 0.1 g l^{-1} streptomycin and 100 000 i.u. l^{-1} penicillin G calcium (pH 7.6). The oocytes were pre-incubated in 1 ml of Leibovitz's L-15 medium only for 0 h (control 0) or 12 h (control 12). Moreover, the oocytes were pre-incubated in 1 ml of Leibovitz's L-15 medium in the presence of DHP (10 ng ml^{-1}), IGF-I (10 μmol ml^{-1}), PmGtH-I (100 ng ml^{-1} or 300 ng ml^{-1}) or PmGtH-II (100 ng ml^{-1} or 300 ng ml^{-1}) for 12 h, washed three times with fresh Leibovitz's L-15 medium, transferred to Leibovitz's L-15 medium containing DHP (10 ng ml^{-1}) and/or actinomycin D (1 μg ml^{-1}; Sigma) and incubated for a further 12 h at 20 °C.

The levels of oocyte maturation were determined by counting the number of oocytes that had undergone germinal vesicle breakdown. The occurrence of germinal vesicle breakdown was used as the criterion of oocyte maturation. After incubation, all the ovarian follicles were stored at −80 °C until used.

Statistical analyses

Data are expressed as means ± S.E.M. Analysis of variance (ANOVA) followed by Duncan's multiple-range tests were applied for statistical analysis.

Isolation of connexin

Two highly conserved regions of human Cx32 (Kumar and Gilula, 1986), rat Cx43 (Beyer et al., 1987) and *Xenopus laevis* Cx30 (Gimlich et al., 1988), Cx38 (Ebihara et al., 1989; Gimlich et al., 1990) and Cx43 (Gimlich et al., 1990) were used to design mixed primers for the polymerase chain reaction (PCR). One of the regions is located at the predicted junction of the first extracellular and second transmembrane domains [5′-TTCCCT(CT)AT(ACT)TC(ACT)CA(CT)(AG)T(CGT)CG-3′] and the other at the junction of the second extracellular and fourth transmembrane domains [5′-GT(CT)TT(CT)TC(ACGT)(AG)TGGG(ACGT)C(GT)(AGT)GA-3′] (Yoshizaki et al., 1994). Total RNA was extracted from incompetent and competent ovaries using a total RNA extraction kit (Pharmacia Biotech, Uppsala, Sweden). Total RNA (5 μg) was used for cDNA synthesis using a first-strand cDNA synthesis kit (Pharmacia Biotech). PCR was carried out using 25 ng of cDNA as template, 10 μmol l^{-1} forward and reverse primers, 200 μmol l^{-1} of each dNTP and *Taq* DNA

polymerase (5 units ml^{-1}; Takara, Japan) in 50 µl of buffer (50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-HCl, pH 9.0, 0.1 % Triton X-100, 1.5 mmol l^{-1} $MgCl_2$). Thirty cycles were conducted, each cycle consisting of 1 min at 94 °C, 45 s at 50 °C and 1 min at 72 °C, except that the first denaturation was carried out for 2 min and the last elongation reaction for 5 min. A sample (5 µl) of each PCR product was electrophoresed in 2 % agarose gels, with the DNA molecular mass marker X (Boehringer Mannheim, Germany) as a reference to estimate the molecular masses of the amplified fragments. The remaining PCR products were electrophoresed in 2 % agarose gels, and fragments were isolated, ligated, cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequenced using a thermo sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Buckinghamshire, UK).

Rapid amplication of cDNA 3′ ends (3′RACE)

Two specific primers (SP) were designed, SP1 (G5′-CAAGGAGCTGGAACACATCA-3′) and SP2 (G5′-AGCTCTGTGGTGGACTTACA-3′). The level of the PCR product (358 bp) of ovarian mRNA increased significantly during the acquisition of oocyte maturational competence. First-strand cDNA synthesis was initiated at the poly(A)$^+$ RNA using the oligo(dT) anchor primer 5′-AACTGGA-AGAATTCGCGGCCGCAGGAAT$_{18}$-3′. Specific cDNA was amplified by PCR using specific primers (SP1 or SP2) that anneal to a region of known sequence and an adaptor primer (5′-AACTGGAAGAATTCGCGGCCGCAG-3′). PCR was carried out with 0.2 µg of cDNA as template, 25 µmol l^{-1} forward and reverse primers, 0.25 mmol l^{-1} of each dNTP and Ex *Taq* polymerase (Takara, Japan) in 50 µl of buffer. Additional controls that amplify dC-tailed cDNA using each primer individually (either the adaptor primer or SP2) were used to identify nonspecific products. PCR was carried out for 30 cycles as follows: 94 °C for 30 s for denaturing, 52 °C for 45 s for primer annealing and 72 °C for 45 s for extension, followed by one cycle of 3 min at 70 °C for extension. The final PCR product was amplified and cloned into pGEM-T Easy Vector and sequenced using the ALF DNA sequencer (Pharmacia Biotech).

Rapid amplication of cDNA 5′ ends (5′RACE)

The cDNA fragment generated by the 3′RACE procedures was used to generate full-length cDNA using a 5′RACE system (version 2.0 kit; Gibco-BRL). Total RNA (1 µg) was reverse-transcribed according to the kit protocol using a gene-specific primer (GSP) (GSP1; 5′-GCAGGGCAGTCTCAGAAA-GCAG-3′) located within the coding sequence. Two further GSPs were designed, GSP2 (5′-GACACTGCCAGC-ATGAACACAGTG-3′) and GSP3 (5′-CTTCTGGTTCTT-GATGTGTTCCAGCTCCTT-3′). The full length of the oligo(dG) anchor primer was included with the kit. The final PCR product was amplified and cloned into pGEM-T Easy Vector and sequenced. The DNA sequence was analyzed using the GENETYX-WIN (Software Development Co., Japan) software package.

Northern blot analysis

To clarify whether isolated mRNA from ovarian follicles incubated with each hormone was differentially expressed specifically during the maturation of oocytes, northern blot analysis was performed. Poly(A)$^+$ RNA was purified using an mRNA purification kit (Oligotex-dT30 Super, Japan). Messenger RNA (5 µg) was electrophoresed according to the formaldehyde gel method (Sambrook et al., 1989) and transferred to nylon membranes (Hybond N$^+$) (Amersham) according to the manufacturer's instructions. The samples were prehybridized at 65 °C in rapid hybridization buffer (Amersham) for 10 min, and then hybridized with the probe at 65 °C for 3 h. The PCR product label used for the oligolabelling kit (Amersham) was [^{32}P]dCTP-labelled cDNA probe. Post-hybridization washing was carried out in 2× standard saline citrate (SSC) for 15 min at 65 °C, in 1× SSC/0.5 % sodium dodecyl sulphate (SDS) for 15 min at 65 °C, and finally in 0.1× SSC/0.5 % SDS for 15 min at 65 °C.

The hybridization signals were analyzed using a FUJIX BAS 1000 bio-imaging analyzer (Fuji Film, Japan). The expression level of each sample was normalized to the red seabream β-actin signal and expressed as percentage of the control level.

Results

One major fragment (358 base pairs) was amplified from the competent oocytes. Homology analyses using the GenBank and EMBL general database searches indicated that the nucleotide sequence of the 358 base pair (bp) cDNA has a high homology with bovine Cx32 (Simonic et al., 1997) (69.1 % identity) and human Cx32 (Kumar and Gilula, 1986) (87.3 % identity). As shown by PCR, levels of amplified cDNA fragments of 358 bp increased simultaneously with the acquisition of oocyte maturational competence (data not shown). The connexin cDNAs generated by the 3′RACE and 5′RACE procedures may be combined to generate a full-length cDNA sequence. The 2400 bp cDNA had an open reading frame of 825 bp that began with the first ATG codon at position 359 and ended with a TGA stop codon at position 1184 (Fig. 1). A putative polyadenylation signal AATAAA (Proudfoot and Brownlee, 1976) occurred at position 2366 (Fig. 1). This open reading frame encodes a polypeptide that contains 275 amino acid residues (Fig. 1) with a predicted molecular mass of 31393 Da. We designated this protein red seabream *P. major* (Pm) Cx31.5 on the basis of the nomenclature system of Beyer et al. (1987).

The GenBank and EMBL databases showed that, among all previously reported connexin genes, PmCx31.5 has the highest homology to human Cx32 (66.3 % similarity and 88.0 % identity) (Simonic et al., 1997) and rat Cx32 (65.3 % similarity and 88.3 % identity) (Kumar and Gilula, 1986) (Fig. 2). Hydropathicity analysis of the predicted amino acid sequences using the method of Kyte and Doolitte (1982) indicated that PmCx31.5 has four major hydrophobic regions and four major hydrophilic regions. Hydrophobic regions predict transmembrane domains, while hydrophilic regions indicate

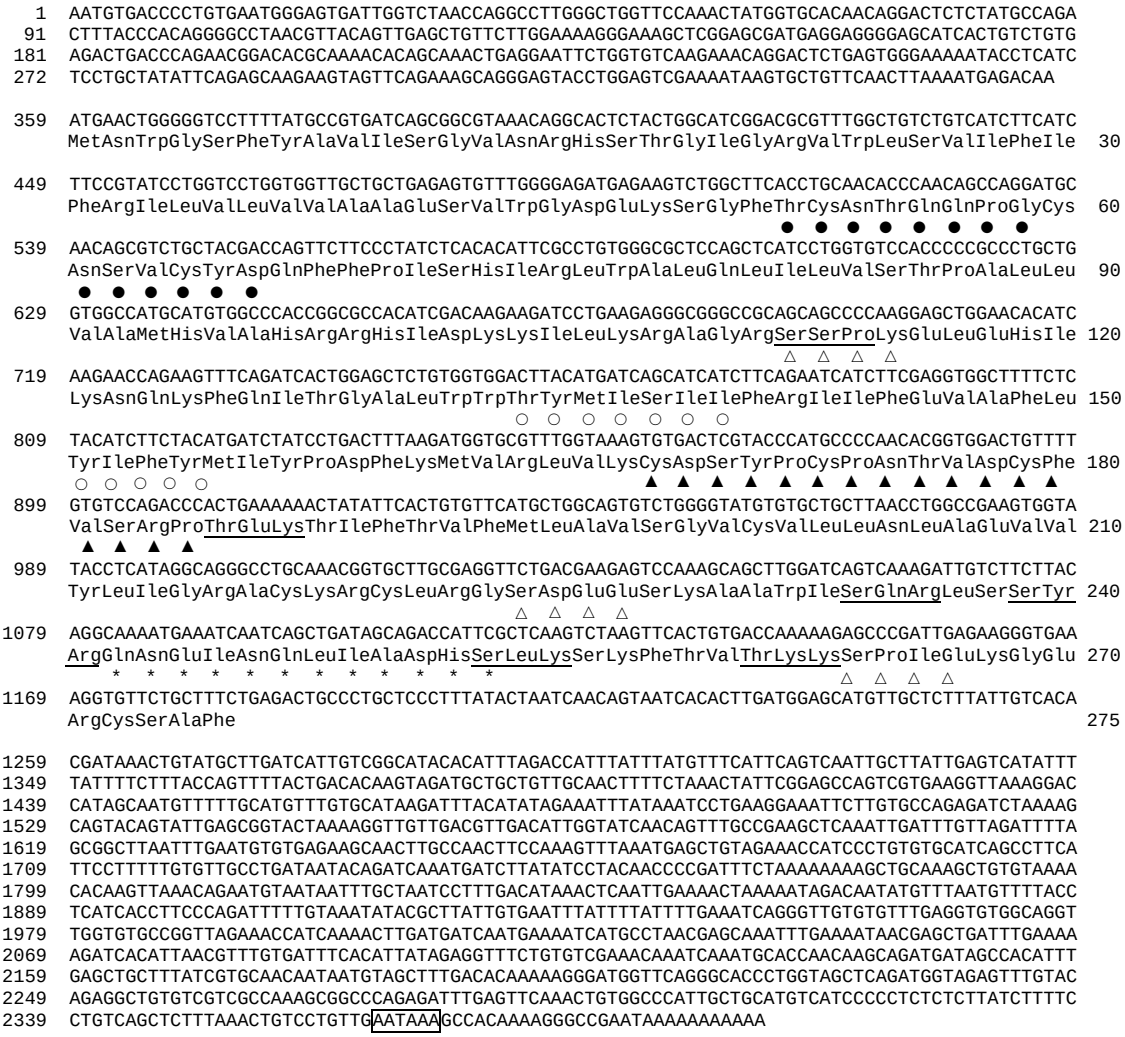

Fig. 1. The base sequence of the red seabream connexin (Cx31.5) cDNA clone and its putative amino acid sequence. The nucleotide number is shown on the left and the amino acid residue number is shown on the right. Filled circles and filled triangles indicate connexin consensus sequences in each extracellular domain. Asterisks indicate a consensus sequence in the C-terminal, cytoplasmic domain. Open circles indicate the predicted amphipathic helix-forming sequence of the third transmembrane domain. Open triangles show the predicted casein protein kinase II phosphorylation sites. Underlined amino acid residues indicate predicted kinase C phosphorylation sites. The polyadenylation signal is boxed. This sequence is available from the DDBJ/EMBL/GenBank nucleotide sequence databases (accession number AB037933).

extracellular or intracellular domains (Fig. 3). Thus, the identify and similarity between the positively established domains of rat Cx32 (Haefliger et al., 1992) and Atlantic croaker Cx32.2 (Yoshizaki et al., 1994) and the corresponding putative domains of PmCx31.5 can be determined individually (Table 1).

With the exception of the intracellular loop and C-terminal domain, all other domains of PmCx31.5 showed relatively high homology with that of Atlantic croaker Cx32.2 (44.2 % identity and 67.6 % similarity for Cx31.5). Connexin consensus sequences containing three cysteine residues were identified in each of the extracellular domains (loops): C(D,N)TXQPGCX_2VCYD in the first loop (Fig. 1; filled circles) and C$X_{3\sim4}$PCX_3(L,I,M,V)(D,E,N)C(F,Y)(L,I,V,M)(S,A)(K,R)P (Yoshizaki et al., 1994) in the second loop (Fig. 1; filled triangles). Further, a consensus sequence found in the C-terminal cytoplasmic domain of most connexins, QNX_{6or9}S (Hoh et al., 1991), was identified in Cx31.5 at amino acid residues 242–253 (Fig. 1; asterisks). The amphipathic helix consensus of the third transmembrane domain, TX_3SX_3(K,R)X_3E (Hoh et al., 1991), was present at positions 134–145 (Fig. 1; open circles). As described above, PmCx31.5 is similar to the connexin family in its general features, and all the consensus sequences are present.

Northern blot analysis of the PmCx31.5 transcript with the β-actin probe as a positive control was carried out using 5 μg of poly(A)$^+$ RNA extracted from ovarian follicles cultured for 12 h in normal medium, DHP (10 ng ml^{-1}), IGF-I (10 μmol ml^{-1}), PmGtH-I (100 ng ml^{-1} or 300 ng ml^{-1}) or PmGtH-II (100 ng ml^{-1} or 300 ng ml^{-1}) (Fig. 4). PmCx31.5 mRNA was strongly expressed in the presence of PmGtH-II (300 ng ml^{-1}), but not in the presence of DHP, IGF-I or PmGtH-I. Fig. 5 shows the effects of control 12, DHP (10 ng ml^{-1}), IGF-I (10 μmol ml^{-1}),

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PmCx31.5     1:M-NWGSFYAVISGVNRHSTGIGRVWLSVIFIFRILVLVVAAESVWGDEKSGFTCNTQQPGCNSVCYDQFFPISHIRLWAL   79
CrCx32.2     1:MGDLGFLSKLLDQVQSHSTVIGKIWMTVLFLFRIMVLGAGAESVWGDEQSDFTCNTQQPGCENVCYDWTFPISHIRFWVL   80
RaCx32.0     1:M-NWTGLYTLLSGVNRHSTAIGRVWLSVIFIFRIMVLVVAAESVWGDEKSSFICNTLQPGCNSVCYDHFFPISHVRLWSL   79
XpCx30.0     1:M-NWAGLYAILSGVNRHSTSIGRIWLSVVFIFRIMVLVAAAESVWGDEKSAFTCNTQQPGCNSVCYDHFFPISHIRLWAL   79
               *           *  *** **  *  * * *** **   ******** * * *** ****  ****  ***** * * *

PmCx31.5    80:QLILVSTPAL--LV-AMHVAHRRHIDKKILKRAGRSSPKEL-EHIK-NQKFQITGALWWTYMISIIFRIIFEVAFLYIFY  154
CrCx32.2    81:QIIFVSTPTLIYLGHAMHIIQQETKLRARLSSPGGSRLCKQPKYTNEQGKVKIKGNLLGSYLTQLVFKIIIEAAFIVGQY  160
RaCx32.0    80:QLILVSTPAL--LV-AMHVAHQQHIEKKMLRLEGHGDPLHL-EEVK-RHKVHISGTLWWTYVISVVFRLLFEAVFMYVFY  154
XpCx30.0    80:QLIIVSTPAL--LV-AMHVAHLQHQEKKELRLSRHVKDQEL-AEVK-KHKVKISGTLWWTYISSVFFRIIFEAAFMYIFY  154
               * * **** *  *  ***           *                   *  * * *   *     *    *  *    *

PmCx31.5   155:MIYPDFKMVRLVKCDSYPCPNTVDCFVSRPTEKTIFTVFMLAVSGVCVLLNLAEVVYLIGRAC-KRCLRGSDEESKAAW-  232
CrCx32.2   161:YLY-GFIMVPMFPCSKKPCPFTVECYMSRPTEKTIFIIFMLVVACVSLLLNVIEVFYLI-CT-RVRCGSRAHSHKITSAE  237
RaCx32.0   155:LLYPGYAMVRLVKCEAFPCPNTVDCFVSRPTEKTVFTVFMLAASGICIILNVAEVVYLIIRACARRAQRRSNPPSRKGSG  234
XpCx30.0   155:LIYPGYSMIRLLKCDAYPCPNTVDCFVSRPTEKTIFTVFMLVASGVCIVLNVAEVFFLIAQACTRRA-RR-H---R-DSG  228
             *    *     *   *****  *  ******* *  ***        **  **  **      *

PmCx31.5   233:ISQRLS-SYRQNEINQLIADH---SLKS-KF-TVTKKSPI-EKGERCSAF                                 275
CrCx32.2   238:NPASLSSPRWPTVEDSLKQNKMNMELETSQSIGGSLDGAKEEKRLLSH--                                 285
RaCx32.0   235:FGHRLSPEYKQNEINKLLSEQDG-SLKDILRRSPGTGAGLAEKSDRCSAC                                 283
XpCx30.0   229:-S--ISKEHQQNEMN-LLIT-GG-S---IIKRS----AG-QEKGDHCSTS                                 264
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Fig. 2. Comparison of the amino acid sequences of red seabream *Pagrus major* (Pm) Cx31.5, rat Cx32, *Xenopus laevis* Cx30 and Atlantic croaker *Micropogonias undulatus* Cx32.2. The PmCx31.5, rat Cx32, *Xenopus laevis* Cx30 and Atlantic croaker Cx32.2 sequences were optimally aligned so as to match identical residues, which are indicated by asterisks. The sequences were extracted from the DDBJ/EMBL/GenBank sequence databases. The connexin sequences (and accession numbers) used for alignment are red seabream Cx31.5 (PmCx31.5) (this paper, AB037933), Atlantic croaker Cx32.2 (CrCx32.2) (L31542), rat Cx32 (RaCx32.0) (X04070) and *Xenopus laevis* Cx30 (XpCx30.0) (Y00791). Gaps in the sequences are indicated as dashes.

Table 1. *Position in the amino acid sequence of predicted topological domains and percentage amino acid sequence identity and similarity of red seabream Cx31.5 with the respective topological domains of Atlantic croaker Cx32.2 and rat Cx32*

		Atlantic croaker Cx32.2		Rat Cx32	
Predicted domain	Amino acid position	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
N	1–19	30.3	63.2	63.2	94.7
TM1	20–40	50.0	89.5	95.2	100
EX1	41–75	75.0	88.2	85.7	94.3
TM2	76–96	43.5	73.9	95.2	100
IN	97–124	18.1	36.2	42.9	85.7
TM3	125–157	21.6	54.5	52.9	94.1
EX2	158–188	50.0	65.6	75.0	87.5
TM4	189–214	50.0	72.0	73.1	92.3
C	215–275	15.9	50.0	41.7	68.1

N, amino-terminal domain (intracellular); TM1,2,3,4, first, second, third and fourth transmembrance domains; EX1,2, first and second extracellular loops; IN, intracellular loop; C, carboxy-terminal domain (intracellular).

Atlantic croaker Cx32.2 and rat Cx32 were chosen for this comparison since their topology has already confirmed.

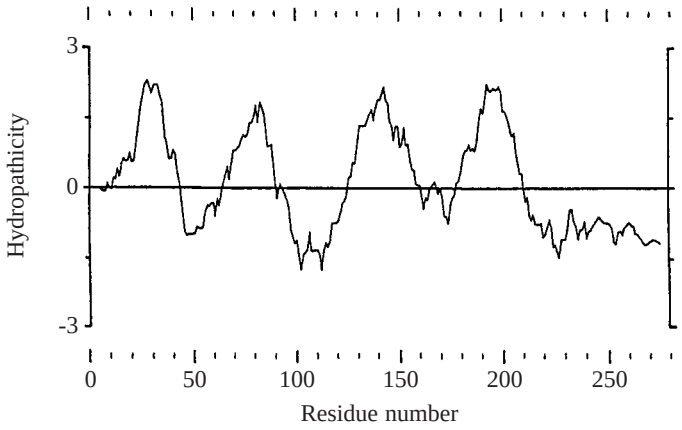

Fig. 3. Hydropathicity plot of red seabream Cx31.5. Hydropathicity values were determined using the method of Kyte and Doolittle (1982) with a window size of 20 residues. The amino acid residue number is shown on the *x*-axis. Negative values are hydrophilic and positive values are hydrophobic.

PmGtH-I (100 ng ml^{-1} or 300 ng ml^{-1}) and PmGtH-II (100 ng ml^{-1} or 300 ng ml^{-1}) on the percentage *in vitro* germinal vesicle breakdown of oocytes and on the PmCx31.5 transcription level. PmGtH-II induced a high level of oocyte germinal vesicle breakdown. Only in the presence of GtH-II (300 ng ml^{-1}) was the transcription level of PmCx31.5 significantly different (P<0.01) from the control value. Northern blot analysis of poly(A)$^+$ RNA (5 μg) extracted from maturationally competent and incompetent ovaries is shown in Fig. 6. PmCx31.5 mRNA was strongly expressed after, but not before, the acquisition of oocyte maturational competence.

Discussion

We have shown that GtH-II induced oocyte maturational competence in the ovarian follicles of the red seabream. Kagawa et al. (1998) reported that GtH-II, but not GtH-I, is involved in the final maturation of red seabream oocytes. Moreover, in the oocytes of the Atlantic croaker, actinomycin D, an inhibitor of RNA synthesis, blocked the induction of

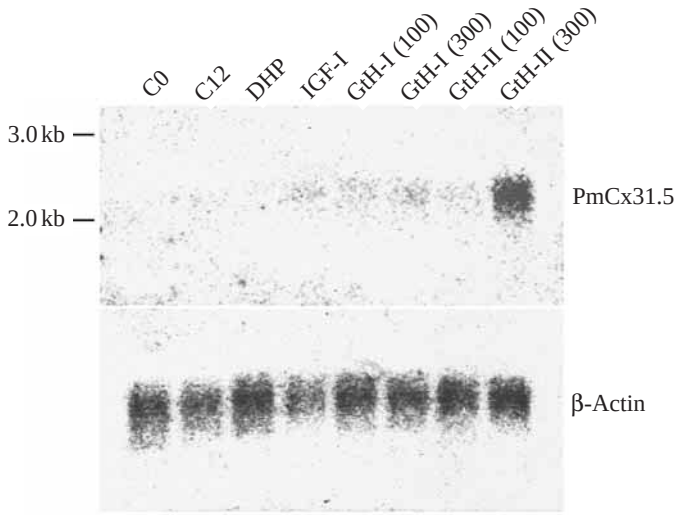

Fig. 4. Effects of 17α,20β-dihydroxy-4-pregnen-3-one (DHP, 10 ng ml^{-1}), human insulin-like growth factor-I (IGF-I, 10 μmol ml^{-1}), red seabream gonadotropin-I (GtH-I, 100 ng ml^{-1} and 300 ng ml^{-1}) and GtH-II (100 ng ml^{-1} and 300 ng ml^{-1}) on red seabream (*Pagrus major*) Cx31.5 (PmCx31.5) transcription level. Northern blot analysis of poly(A)$^+$ RNA (5 μg) extracted from ovarian follicles and hybridized with PmCx31.5 and β-actin probes. The positions of RNA molecular mass markers are shown on the left. C0, control; C12, incubated in normal medium for 12 h.

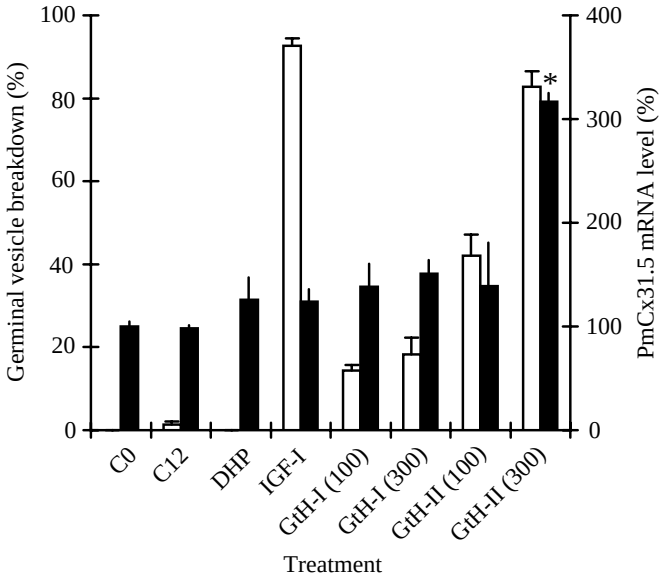

Fig. 5. Effects of 17α,20β-dihydroxy-4-pregnen-3-one (DHP, 10 ng ml^{-1}), human insulin-like growth factor-I (IGF-I, 10 μmol ml^{-1}), red seabream gonadotropin-I (GtH-I, 100 ng ml^{-1} and 300 ng ml^{-1}) and GtH-II (100 ng ml^{-1} and 300 ng ml^{-1}) on percentage *in vitro* germinal vesicle breakdown and red seabream (*Pagrus major*) Cx31.5 (PmCx31.5) transcription level. mRNA (5 μg) [poly(A)$^+$ RNA] extracted from each group of hormone-treated ovarian follicles was hybridized with PmCx31.5 and β-actin probes. The expression level of each sample was normalized to the red seabream β-actin signal, and is expressed as percentage of the control 0 (C0) level. An asterisk indicates a significant difference compared with the respective C0 value (P<0.01). Open columns, germinal vesicle breakdown (%); filled columns, PmCx31.5 mRNA level. C12, incubated in normal medium for 12 h. Values are means + S.E.M. (N=3).

oocyte maturational competence by gonadotropin (Patino and Thomas, 1990). Kagawa et al. (1994) reported that hCG induces maturational competence in red seabream oocytes *via de novo* synthesis of RNA and protein. Newly synthesized proteins are therefore probably involved in hCG (or GtH-II)-induced oocyte maturation. However, if hCG (or GtH-II) directly stimulates an increase in oocyte maturational competence in ovarian follicles, this increase should not require *de novo* protein synthesis. In the present study, connexin cDNA was expressed during the acquisition of oocyte maturational competence in ovarian follicles after treatment with GtH-II.

This study demonstrated that the amount of connexin mRNA in the gap junctions of ovarian follicles increases as a result of treatment with GtH-II. We are not sure what role the gap junction plays in the function of ovarian follicles. Electron microscopy has demonstrated that gap junctions form between microvilli between the oocyte and granulosa cells of *Xenopus laevis* (Gimlich et al., 1988; Ebihara et al., 1989), medaka (*Oryzias latipes*) (Iwamatsu and Ohta, 1981) and zebrafish (*Brachydanio rerio*) (Kessel et al., 1985). Furthermore, gonadotropin treatment increases the number of gap junctions in *Xenopus laevis* (Yoshizaki and Patino, 1995) and Atlantic croaker (Yoshizaki et al., 1994) ovarian follicles. Reports suggest that the intercellular movement of Lucifer Yellow from oocytes to granulosa cells is promoted by gonadotropin in

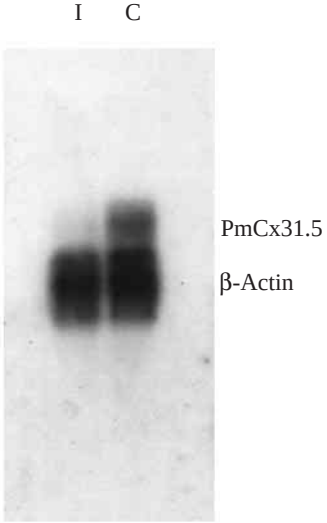

Fig. 6. Northern blot analysis of poly(A)$^+$ RNA extracted from incompetent (I) and competent (C) ovaries. Incompetent and competent ovaries were collected at 08:00 h and 16:00 h, respectively. Poly(A)$^+$ RNA (5 μg) was used for electrophoresis. After transfer to the membrane, the duplicated series of blots were separately used for probing with [^{32}P]dCTP-labelled red seabream (*Pagrus major*) Cx31.5 (PmCx31.5) cDNA and β-actin.

Xenopus laevis (Browne et al., 1979) and *Bufo arenarum* (Villecco et al., 1996).

The role of the gap junction in the maturation of oocytes can be inferred from the observation that only GtH-II induces increases in PmCx31.5 mRNA levels, while GtH-I and DHP do not. Moreover, Yoshizaki et al. (1994) reported that levels of mRNA transcripts of Atlantic croaker Cx32.2 were increased when hCG treatment was used to stimulate the acquisition of oocyte maturational competence. This phenomenon was independent of the production of maturation-inducing steroid, which has the same effect as hCG on the acquisition of oocyte maturation competence in the Atlantic croaker (Chang et al., 1999). In the present study, PmCx31.5 showed a similar expression pattern to Atlantic croaker Cx32.2, which is also expressed after hCG (GtH-II) treatment.

GtH-II was found to increase the number of gap junctions between the oocyte and granulosa cell *via* increased levels of PmCx31.5, leading to the acquisition of oocyte maturational competence. However, although IGF-I effectively induces germinal vesicle breakdown, it does not affect the level of PmCx31.5 transcripts (Figs 4, 5). It would appear, therefore, that the induction of oocyte maturational competence by treatment with IGF-I results from an action different from that of gonadotropin. This agrees with a previous report (Kagawa et al., 1994) that IGF-I transcription is not involved in the acquisition of oocyte maturational competence. The mechanism for the induction of oocyte maturational competence by IGF-I remains unexplained.

It is noteworthy that PmCx31.5 possesses protein kinase C (PKC) phosphorylation sites, (S,T)X(R,K) (Kishimoto et al., 1985; Woodget et al., 1986), and casein protein kinase II phosphorylation sites, (S,T)X_2(D,E) (Pinna, 1990) (Fig. 1). These kinases regulate phosphorylation in many systems (Hoh et al., 1991). Takeda et al. (1987) reported that rat Cx32 was phosphorylated by PKC *in vitro*. Atlantic croaker Cx32.2 also has PKC phosphorylation sites, and the increase in ovarian Cx32.2 mRNA production coincides with the induction of oocyte maturational competence (Yoshizaki et al., 1994). Moreover, phorbol 12-myristate 13-acetate (PMA), a PKC activator, inhibits the induction of oocyte maturational competence by gonadotropin (Stagg and Fletcher, 1990; Chang et al., 1999). It is conceivable, therefore, that the cyclic AMP/protein kinase A (PKA) transduction pathway is involved in the activation of the Cx31.5 gene by gonadotropin in the red seabream, particularly since cyclic AMP response elements have been identified in the 5′ upstream region (Miller et al., 1988; Yoshizaki et al., 1994). In the nibe (*Nibea mitsukuri*), oocyte maturation is induced through the activation of PKA (G. Yoshizaki, personal communication). Furthermore, PKC acutely inhibits and PKA acutely enhances cell–cell junctional coupling (Stagg and Fletcher, 1990). In our study, a northern blot analysis was carried out using 5 μg of poly(A)$^+$ RNA extracted from incompetent and competent ovaries (Fig. 6). PmCx31.5 mRNA was strongly expressed after, but not before, the acquisition of oocyte maturational competence. These results suggest that the production of Cx31.5 in the red seabream ovary is related to the maturation of ovarian follicles to produce competent oocytes.

In summary, this study found that the induction of oocyte maturational competence by GtH-II requires *de novo* synthesis of ovarian RNA and protein and coincides with an increase in the production of ovarian PmCx31.5 mRNA and the establishment of granulosa cell–oocyte gap junctions in fully grown ovarian follicles (York et al., 1993). Thus, it is possible that PmCx31.5 is the protein synthesized during the gonadotropin-induced acquisition of maturational competence in red seabream ovaries. We have not determined the tissue distribution of PmCx31.5 gene expression, nor have the mechanisms of PmCx31.5 activation with hCG treatment been addressed in the present study. Moreover, the physiological roles of PmCx31.5 in the acquisition of oocyte maturational competence were not investigated. Further studies are necessary to identify the second-messenger systems that regulate the PmCx31.5 gene under the influence of gonadotropin in the ovary.

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References

Beyer, E. C., Paul, D. L. and Goodenough, D. A. (1987). Connexin 43: A protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* **105**, 2631–2629.

Beyer, E. C., Paul, D. L. and Goodenough, D. A. (1990). Connexin family of gap junction proteins. *J. Membr. Biol.* **116**, 187–194.

Browne, C. L., Wiley, H. S. and Dumont, J. N. (1979). Oocyte–follicle cell gap junctions in *Xenopus laevis* and the effects of gonadotropin on their permeability. *Science* **203**, 182–183.

Bruzzone, R., White, T. W. and Paul, D. W. (1996). Connections with connexins: the molecular basis of direct intercellular signaling. *Eur. J. Biochem.* **238**, 1–27.

Cerda, J. L., Peetrino, T. R. and Wallace, R. A. (1993). Functional heterologous gap junctions in *Fundulus* ovarian follicles maintain meiotic arrest and permit hydration during oocyte maturation. *Dev. Biol.* **160**, 228–235.

Chang, X., Patino, R., Thomas, P. and Yoshizaki, G. (1999). Developmental and kinase-dependent regulation of ovarian connexin mRNA and oocyte maturational competence in Atlantic croaker. *Gen. Comp. Endocr.* **114**, 330–339.

Degani, G. and Boker, R. (1992). Sensitivity to maturation-inducing steroids and gonadotropin in the oocytes of blue gourami, *Trichogaster trichopterus* (Anabantidae, Pallas, 1770). *Gen. Comp. Endocr.* **85**, 430–439.

Ebihara, L., Beyer, E. C., Swenson, K. I., Paul, D. L. and Goodenough, D. A. (1989). Cloning and expression of a *Xenopus* embryonic gap junction protein. *Science* **243**, 1194–1195.

Gimlich, R. L., Kumar, N. M. and Gilula, N. B. (1988). Sequence

and developmental expression of mRNA coding for a gap junction protein in *Xenopus*. *J. Cell Biol.* **107**, 1065–1073.

Gimlich, R. L., Kumar, N. M. and Gilula, N. B. (1990). Differential regulation of the levels of three gap junction mRNAs in *Xenopus* embryos. *J. Cell Biol.* **110**, 597–605.

Grazul-Bilska, A. T., Reynolds, L. P. and Redmer, D. A. (1997). Gap junctions in the ovaries. *Biol. Reprod.* **57**, 947–957.

Haefliger, J. A., Bruzzone, R., Jenkins, N. A., Gilbert, D. J., Copeland, N. G. and Paul, D. L. (1992). Four novel numbers of the Connexin family of gap junction proteins. Molecular cloning, expression and chromosome mapping. *J. Biol. Chem.* **267**, 2057–2064.

Hertzberg, E. L., Spray, D. C. and Bennett, M. V. L. (1985). Reduction of gap junctional conductance by microinjection of antibodies against the 27 kDa liver gap junction polypeptide. *Proc. Natl. Acad. Sci. USA* **82**, 2412–2416.

Hoh, J. H., John, S. A. and Revel, J. P. (1991). Molecular cloning and characterization of a new member of the gap junction gene family, connexin-31. *J. Biol. Chem.* **266**, 6524–6531.

Iwamatsu, T. and Ohta, T. (1981). On a relationship between oocytes and follicle cells around the time of ovulation in the medaka, *Oryzias latipes*. *Annot. Zool. Japon* **54**, 17–29.

Kagawa, H., Kobayashi, M., Hasegawa, Y. and Aida, K. (1994). Insulin and insulin-like growth factor-I and -II induce final maturation of oocytes of red seabream, *Pagrus major*, *in vitro*. *Gen. Comp. Endocr.* **95**, 293–300.

Kagawa, H., Tanaka, H., Okuzawa, K. and Kobayashi, M. (1998). GTH-II but not GTH-I induces final maturation and the development of maturational competence of oocytes of red seabream *in vitro*. *Gen. Comp. Endocr.* **112**, 80–88.

Kessel, R. G., Tung, H. N., Roberts, R. and Beams, H. W. (1985). The presence and distribution of gap junctions in the oocyte–follicle cell complex of the zebrafish, *Brachydanio rerio*. *J. Submicrosc. Cytol.* **17**, 239–253.

Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y. and Nishizuka, Y. (1985). Studies on the phosphorylation of myelin basic protein by protein kinase C and adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **260**, 12492–12499.

Kumar, N. M. and Gilula, N. B. (1986). Cloning and characterization of human and rat liver cDNAs coding for a gap junction protein. *J. Cell Biol.* **103**, 767–776.

Kyte, J. and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132.

Matsuyama, M., Adachi, S., Nagahama, Y. and Matsuura, S. (1988). Diurnal rhythm of oocyte development and plasma steroid hormone levels in the female red seabream, *Pagrus major* during the spawning season. *Aquaculture* **73**, 357–372.

Miller, T., Dahl, G. and Werner, R. (1988). Structure of a gap junction gene: rat connexin-32. *Biosci. Rep.* **8**, 445–464.

Patino, R. and Thomas, P. (1990). Effects of gonadotropin on ovarian intrafollicular processes during the development of oocytes maturational competence in a teleost, the Atlantic croaker: Evidence for two distinct stages of gonadotropic control of final oocyte maturation. *Biol. Reprod.* **43**, 818–827.

Pinna, L. A. (1990). Casein kinase 2: An ‘eminence grise’ in cellular regulation? *Biochim. Biophys. Acta* **1054**, 267–284.

Proudfoot, N. J. and Brownlee, G. G. (1976). 3′ Non-coding region sequences in eukaryotic messenger RNA. *Nature* **263**, 211–214.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Electrophoresis of RNA through gels containing formaldehyde. In *Molecular Cloning: A Laboratory Manual*, vol. 1 (ed. N. Ford, C. Nolan and M. Ferguson), pp. 7.43–7.45. New York: Cold Spring Harbor Laboratory Press.

Simonic, T., Duga, S., Negri, A., Tedeschi, G., Malcovati, M., Tenchini, M. L. and Ronchi, S. (1997). cDNA cloning and expression of the flavoprotein D-aspartate oxidase from bovine kidney cortex. *Biochem. J.* **322**, 729–735.

Spray, D. C., Harris, A. L. and Bennett, N. V. L. (1981). Gap junction conductance is a simple and sensitive function of intracellular pH. *Science* **211**, 712–715.

Stagg, R. B. and Fletcher, W. H. (1990). The hormone-induced regulation of contact-dependent cell–cell communication by phosphorylation. *Endocr. Rev.* **11**, 302–325.

Takeda, A., Hashimoto, E., Yamamura, H. and Shimazu, T. (1987). Phosphorylation of liver gap junction protein-by-protein kinase C. *FEBS Lett.* **210**, 169–172.

Tanaka, H., Kagawa, H., Okuzawa, K. and Hirose, K. (1993). Purification of gonadotropins (PmGTH-I and -II) from red seabream, *Pagrus major* and development of a homologous radioimmunoassay for PmGTH-II. *Fish Physiol. Biochem.* **10**, 409–418.

Teranishi, T., Negishi, K. and Kato, S. (1983). Dopamine modulates S-potential amplitude and dye-coupling between external horizontal cell in carp retina. *Nature* **301**, 243–246.

Villecco, E. I., Aybar, M. J., Sanchez, S. S. and Sanchezriera, A. N. (1996). Heterologous gap junctions between oocyte and follicle cells in *Bufo arenarum*: hormonal effects on their permeability and potential role in meiotic arrest. *J. Exp. Zool.* **276**, 76–85.

Woodget, J. R., Gould, K. L. and Hunter, T. (1986). Substrate specificity of protein kinase C. Use of synthetic peptides corresponding to physiological sites as probes for substrate recognition requirements. *Eur. J. Biochem.* **161**, 177–184.

York, W. S., Patino, R. and Thomas, P. (1993). Ultrastructural changes in follicle cell–oocyte associations during development and maturation of the ovarian follicle in Atlantic croaker. *Gen. Comp. Endocr.* **92**, 402–418.

Yoshizaki, G. and Patino, R. (1995). Molecular cloning, tissue distribution and hormonal control in the ovary of Cx41 mRNA, a novel *Xenopus* connexin gene transcript. *Mol. Reprod. Dev.* **42**, 7–18.

Yoshizaki, G., Patino, R. and Thomas, P. (1994). Connexin messenger ribonucleic acids in the ovary of Atlantic croaker: Molecular cloning and characterization, hormonal control and correlation with appearance of oocyte maturational competence. *Biol. Reprod.* **51**, 493–503.