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Insulin-Like Growth Factor-Binding Protein (IGFBP)-1, -2, -3, -4, -5, and -6 and IGFBP-Related Protein 1 during Rainbow Trout Postvitellogenesis and Oocyte Maturation: Molecular Characterization, Expression Profiles, and Hormonal Regulation

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In the present study we report the full coding sequence of rainbow trout IGF-binding protein-1 (IGFBP1), -2, -3, -5, and -6 and IGFBP-related protein-1 (IGFBP-rP1) mRNAs as well as the partial coding sequence of IGFBP-4 mRNA. To our knowledge, this is the first report of IGFBP4, IGFBP6, and IGFBP-rP1 in a nonmammalian species. The tissue distribution of all mRNAs was studied, and the ovarian expression profiles of IGFBP2 to -6 and IGFBP-rP1 between late vitellogenesis and oocyte maturation were characterized. In addition, *in vitro* hormonal regulation by the maturation-inducing steroid 17,20β-dihydroxy-4-pregnen-3-one (17,20βP), gonadotropin, and estradiol were studied. We observed that besides IGFBP1, which was only found in liver, IGFBP2 to -6 and IGFPB-rP1 were expressed in the preovulatory ovary. IGFBP3 was also detected in liver, trunk, kidney, skin, and gills, whereas IGFBP2 to -6 and IGFBP-rP1 exhibited a wider tissue distribution. In the preovulatory ovary, IGFBP3 was strongly down-regulated during the postvitellogenesis period, whereas IGFBP5 exhibited a limited up-regulation. In addition, IGFBP6 and IGFBP-rP1 were up-regulated during oocyte maturation. Hormonal regulation data indicated that all ovarian IGFBPs and IGFBP-rP1 transcripts are regulated under gonadotropic stimulation at a concentration that induced 100% oocyte maturation. In addition, IGFBP2 to -5 transcripts are regulated by 17,20βP and estradiol. Together, our observations strongly suggest that during final oocyte maturation, a down-regulation of IGFBP3, -4, and -5 occurs in the oocyte in response to gonadotropic and 17,20βP (IGFBP3 and -5) stimulation, whereas an up-regulation of IGFBP2 and -6 occurs in follicular layers or extrafollicular tissue in response to gonadotropic stimulation. (*Endocrinology* 147: 2399–2410, 2006)

THE IGF SYSTEM includes IGFs (IGF-I and IGF-II), type I and type II receptors (IGF-RI and IGF-RII), and IGF-binding proteins (IGFBPs). There are six well-characterized mammalian IGFBPs, designed IGFBP1 to IGFBP6 (1). Resulting from their higher affinity for IGFs than that of the IGF-RI itself, these binding proteins act not only as carriers of IGFs, but also function as modulators of IGF's availability and activity. In addition to this IGF-dependent action, there are numerous data supporting the importance of IGFBPs for cell growth in an IGF-independent manner (2). In addition to the six IGFBP forms, closely related cysteine-rich proteins sharing structural similarities with IGFBPs have been described in mammals (1). These proteins are referred to as IGFBP-related proteins (IGFBP-rP), but despite their sequence similarities with IGFBPs, they exhibit a low affinity for IGFs, suggesting that they act in an IGF-independent manner (1). To date, nine IGFBP-rPs have been identified in humans and have been implicated in the regulation of cell proliferation and differentiation (1).

In fish, it has been know for several years that the IGF system is implicated in follicular maturational competence acquisition and oocyte maturation. IGFs have been observed to induce intrafollicular oocyte maturation *in vitro*, probably by direct action at the oocyte level, as observed in sea bream and *Fundulus* (3, 4). However, these factors could also act through modulation of maturation-inducing steroid, 17,20β-dihydroxy-4-pregnen-3-one (17,20βP), synthesis, as suggested by the effect of IGF-I on 17,20βP production by granulosa cells of coho salmon (5, 6) or rainbow trout (7). More recently, it was shown in rainbow trout that IGF-I and IGF-II mRNA levels progressively increase in the ovary during competence acquisition and oocyte maturation (8, 9). Preliminary evidence also showed that IGFBP2 transcript was present in the rainbow trout preovulatory ovary (9). However, the amount of information on IGFBP expression in the preovulatory ovary is extremely limited, and our comprehension of IGF system involvement in late oogenetic steps thus remains incomplete. Recently, IGFBP1, -2, -3, and -5 have been cloned from several fish species (10–15). However, the six forms were never identified in the same species. In addition, some IGFBP proteins have been purified and par-

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Abbreviations: aa, Amino acid; CDS, complete coding sequence; Ct, cycle threshold; E2, estradiol; EST, expressed sequence tag; GVBD, germinal vesicle breakdown; IGFBP-rP1, IGF-binding protein-related protein-1; IGF-RI, type I IGF receptor; 17,20βP, 17,20β-dihydroxy-4-pregnen-3-one; rt, rainbow trout.

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tially sequenced from rainbow trout (16) and chinook salmon (17). Finally, despite this advance in IGFBP research in fish over the past few years, no data are available to date on IGFBP-rP or IGFBP4 and -6 in lower vertebrates. Therefore, the present study aimed at 1) characterizing the cDNA sequence of the six forms of rainbow trout (rt) IGFBPs, 2) identifying the cDNA sequence of any existing IGFBP-rP, 3) studying the tissue distribution of identified IGFBP and IGFBP-rP transcripts, 4) studying the expression profiles of all identified cDNAs in rainbow trout preovulatory ovary, and 5) studying the regulation of those transcripts by the hormones involved in the control of follicular competence acquisition and oocyte maturation: maturing gonadotropin, the natural maturation-inducing steroid 17,20βP, and estradiol (E2).

Materials and Methods

Animal and tissue collection

Investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare. Rainbow trout (*Oncorhynchus mykiss*) from an autumn-spawning strain were obtained from an experimental fish farm (Peima, Sizun, France) approximately 1 month before spawning. Female rainbow trout exhibit a group-synchronous follicular development, leading to the production of gametes once a year, in the fall. Fish were held during spawning season in a recirculated water system at 12 C under a natural photoperiod (Scribe, Rennes, France) until tissue collection. Fish were fed to satiety using commercial trout pellets. Ovaries were sampled during late vitellogenesis (3–4 wk before expected spawning; n = 6), after vitellogenesis (before oocyte maturation, but during the spawning period; n = 6), and during oocyte maturation (n = 6). Late vitellogenic samples were obtained 10 d after transporting the fish into our experimental facilities. Postvitellogenic samples were obtained 2 wk after late vitellogenic samples. Maturing samples were obtained 2 wk after vitellogenic samples. For tissue collection, trout were deeply anesthetized with 2-phenoxyethanol (10 mg/ml water), killed by a blow on the head, and bled by gill arch section. Ovaries were then dissected out of the body cavity under sterile conditions. Ovarian aliquots were frozen in liquid nitrogen and stored at −80 C until RNA extraction. For the tissue distribution study, different tissues were sampled from a postvitellogenic female. Testis samples were also obtained from three different males at three different stages (I, III, and IV) (18).

Isolation of IGFBP cDNAs

Relevant rainbow trout expressed sequence tags (ESTs) were identified using a reciprocal blast search strategy. A tblastn search was performed against all rainbow trout ESTs available in dbEST (19) using zebrafish (*CAC44453*, *AAF23123*, and *AAM51549*) or human (*AAH16041*, *AAH11453*, and *AAH05007*) IGFBP amino acid sequences available in GenBank. The corresponding clones were obtained from the Institut National de la Recherche Agronomic Agenae program resource center (Jouy-en-Josas, France) (20) and fully sequenced in both directions. To obtain complete coding sequences (CDS), the missing 5′ part of the cDNA was obtained by PCR using cDNA rainbow trout ovary (21) and fry cDNA libraries as a template (Uni-ZAP Custom cDNA Library; Stratagene, La Jolla, CA). The reverse primer was designed to be specific for each clone, and the forward primer was specific to the vector (T3 or BK reverse primer depending on the library). PCR amplification was performed in a 100-μl volume using PCR Master Mix (M7502, Promega Corp., Charbonnières, France) and 40 cycles were run (95 C for 40 sec, 60 C for 1 min, and 72 C for 2 min).

To obtain rtIGFBP1 cDNA, primers were designed based on the salmon sequence (*AY662657*; forward primer, 5′-TCTACACCTGAAGATTCGAG-3′; reverse primer, 5′-TGGGTCAGAGTGTCCATGAT-3′). PCR amplification was performed in a 100-μl volume using PCR Master Mix (Promega Corp.), and 35 cycles were run (95 C for 40 sec, 55 C for 1 min, and 72 C for 2 min) using liver cDNA as a template. All PCR products were cloned into the pGEM-T easy vector (Promega), and corresponding inserts were sequenced by the dye termination method (ABI PRISM 310; Applied Biosystems, Foster City, CA).

Structural and phylogenetic tree analyses

Predictions of potential *N*-glycosylation and phosphorylation sites were performed using the prediction server of the Center for Biological Sequence Analysis (www.cbs.dtu.dk) based on the deduced amino acid sequences. Multiple amino acid sequence alignments were constructed using ClustalW software. The amino acid sequences were subjected to ClustalW analysis to construct a phylogenetic tree using a neighbor-joining method. TreeView software was used to prepare a graphical view of the phylogenetic tree (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

RNA extraction and RT

RNA extraction and RT were performed as previously described with minor modifications (9). Briefly, ovarian tissue was homogenized in TRIzol reagent (Invitrogen, Cergy Pontoise, France) at a ratio of 100 mg/ml reagent. Total RNA was extracted using the TRIzol procedure and resuspended in water. Three micrograms of total RNA was reverse transcribed using 200 U Moloney murine leukemia virus reverse transcriptase (Promega Corp.) and 1.25 μg random hexamers (Promega Corp.) according to the manufacturer's instructions. RNA and deoxyNTPs were denatured for 6 min at 70 C, then chilled on ice for 5 min before RT Master Mix was added. RT was performed at 37 C for 1 h and 15 min, followed by a 15-min incubation step at 70 C. Control reactions were run without reverse transcriptase and were used as negative real-time PCR controls.

Real-time PCR

Real-time PCR was performed using an I-Cycler IQ (Bio-Rad Laboratories, Hercules, CA) as previously described (9). RT products were diluted to 1:25, and 5 μl was used for each real-time PCR. Each RT product was run in triplicate. Real-time PCR was performed using a real-time PCR kit provided with a SYBR Green fluorophore (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions and using 600 nM of each primer. After a 2-min incubation at 50 C and a 10-min incubation at 95 C, amplification was performed using the following cycle: 95 C for 20 sec, and 60 or 62 C for 1 min, 40 times. The relative abundance of target cDNA within a sample set was calculated from a serially diluted (standard curve) ovarian cDNA pool using I-Cycler IQ software (Bio-Rad). Before analysis, real-time PCR data were normalized using 18S transcript abundance in the samples. After amplification, a fusion curve was obtained using the following protocol: 10 sec of holding, followed by a 0.5 C increase, repeated 80 times and starting at 55 C. Primer sequences are shown in Table 1.

In vitro hormonal regulation

Full-grown postvitellogenic ovarian follicles were isolated and incubated *in vitro* as previously described (9). Briefly, 25 follicles originating from the same ovary were incubated in 3 ml mineral medium in six-well culture plates. Follicles were incubated in presence of partially purified gonadotropin (23 or 188 ng proteins/ml) (8, 9), 17,20βP (40 ng/ml) and E2 (1 μg/ml). Partially purified gonadotropins were obtained from an affinity chromatography on a concanavalin A-Sepharose of a pool of salmon pituitaries sampled during the spawning season, as previously described (22). This gonadotropin fraction was previously used, and a concentration of 188 ng/ml was found to be efficient in inducing 100% *in vitro* oocyte maturation in most females assayed (8, 9). Similarly, a concentration of 40 ng/ml 17,20βP was previously used to successfully induce *in vitro* oocyte maturation in rainbow trout (23). In addition, a dose of 1 μg/ml E2 was previously found to significantly inhibit oocyte maturation in rainbow trout (24). After a 20-h incubation, follicles were removed and processed for RNA extraction. For each hormonal treatment, three wells (25 follicles each) were used for RNA extraction from intact follicles whereas three other wells were used for RNA extraction from deyolked follicles. All follicles originating from one well were deyolked as previously described by firmly pressing the tissue between

TABLE 1. Primers used for real-time PCR (qPCR) and tissue distribution (t-dist) studies

Gene	GenBank	Study	Forward	Reverse
IGFBP1	DQ190460	qPCR	AGTTCACCAACTTCTACCTACC	GACGACTCACACTGCTTGGC
		t-dist	GGATTATATAAGAAGTTGACCCTGCT	AGCTCCAGCTGGCACTCTAA
IGFBP2	DQ146968	qPCR	GTGCTGGAGAGGATATCTAAGA	AGACATCTTACACTGTTTGAGGT
		t-dist	GGCAAGCAGATTCAGTGTCA	GCGTTTACACACGCAGAAAA
IGFBP3	DQ146966	qPCR	TTCCATGATAACAGGGGACATG	GACCGTGGGTGGACATGTGG
		t-dist	GCCTATTGCTCGGAGACTTG	AGAAACCCATGCACATGACA
IGFBP4	DQ146967	qPCR	TGTCGTGCTGAGCTGCAGAG	TGGCTGGCACTGCTTGGCAT
		t-dist	ACGATGAGACCATCCCTGAG	TGTTGTTTGCCACCTGTCAT
IGFBP5	DQ206713	qPCR	ACTTCACGCGCTTCTCCATGGCA	CGAGACTCATGATCTATGGGTGGA
		t-dist	TTGTCGGTCAATTTGTTCCA	CAAGCAGGTTCCCCTATTCA
IGFBP6	DQ190459	qPCR	GCTCAATAGTGTTCTGCGTGG	CTTGGAGGAACGACACTGCTT
		t-dist	TACCATGCGATGGAGATTGA	TGCTCAGATGTGTGTGGTGA
IGFBP-rP1	DQ146965	qPCR	GCTCCGATGGAGTGACCTATA	ACAATGACAGGTGCTGTTGCG
		t-dist	TCGCTGTGGTCTTATCGTTG	GGATGCTAATGGTGGCTGTT
18S	AF308535	qPCR	CGGAGGTTCGAAGACGATCA	TCGCTAGTTGGCATCGTTTAT
EF1α	AF498320	t-dist	AGCGCAATCAGCCTGAGAGGTA	GCTGGACAAGCTGAAGGCTGAG

Forward and reverse primer sequences and GenBank accession numbers of rainbow trout IGFBP1 to -6, IGFBP-rP1, EF1α, and 18S genes are shown.

two mesh screens under constant cold mineral medium aspersion (25). Total RNA extracted from intact follicles will include the RNA present in full-grown oocytes, follicular walls (granulosa and thecal cells), as well as remaining adjacent ovarian tissue that putatively contains gonial cells, previtellogenic follicles, and connective tissue. In contrast, RNA extracted from deyolked follicles does not contain all the RNA that would be present in full-grown oocytes. In addition, two wells were incubated for 60 h to assess germinal vesicle breakdown occurrence.

Statistical analysis

Statistical analyses were performed using Statistica 7.0 software (Statsoft, Tulsa, OK). Differences between ovarian developments stages were analyzed by ANOVA, followed by Duncan's test (see Fig. 5). Hormonal regulation data (see Fig. 6) were analyzed using nonparametric *U* tests.

Results

Molecular cloning of rtIGFBPs cDNA

By searching rainbow trout ESTs in the dbEST (19) database, six ESTs (*BX315314*, *BX320152*, *BX867870*, *CX246581*, *CA347407*, and *BX870849*) that displayed significant homologies with members of the IGFBP family were identified. Except for one (*CX246581*), all ESTs exhibited a partial CDS. After PCR screening of cDNA libraries, we were able to obtain the complete coding sequence for all rtIGFBPs except IGFBP4. To obtain rtIGFBP1 cDNA, PCR was performed using primers designed from the salmon IGFBP1 sequence (*AY662657*). After cloning and sequencing, the unique 735-bp PCR product obtained corresponded to the full CDS of rtIGFBP1 cDNA.

IGFPB1

The nucleotide sequence of rtIGFBP1 (*DQ190460*) was 735 bp in length with an open reading frame encoding for a 245-amino acid (aa) protein (Fig. 1A). The encoded protein had an estimated molecular mass of 24 kDa and contained 18 cysteine residues. The amino acid sequence was compared with human IGFBPs and was found to be most similar to IGFBP1 (36% identity; Fig. 1B). It also exhibited a high homology with human IGFBP4 (34%). Furthermore, this sequence exhibited 99% and 58% identity with cognate salmon and zebrafish IGFBP1, respectively. Similar to salmon and zebrafish IGFBP1, rtIGFBP1 did not have the RGD integrin recognition sequence found in all mammalian IGFBP1 (26).

IGFBP2

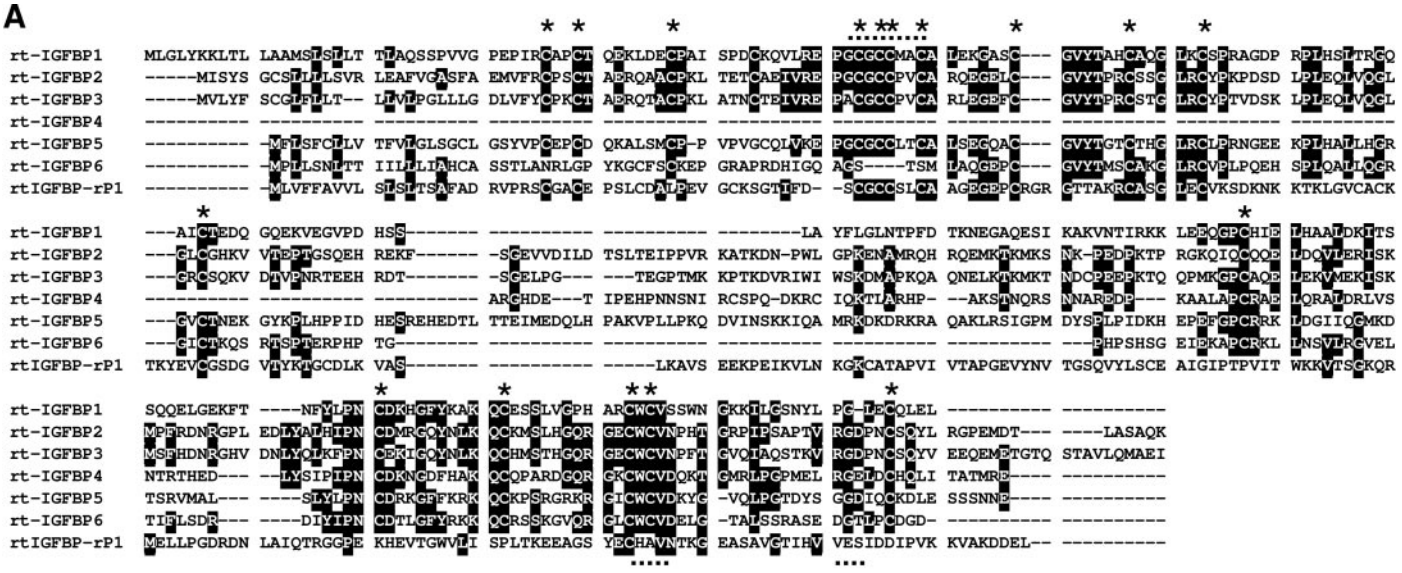
The nucleotide sequence of rtIGFBP2 (*DQ146968*) was 1941 bp in length with an open reading frame coding for 283 aa, including 18 cysteines residues (Fig. 1A). The rtIGFBP2 sequence did not contain any *N*-glycosylation site and had an estimated molecular mass of 28.7 kDa. The rtIGFBP2 as sequence was 73% and 44% identical with cognate zebrafish and human IGFBP2, respectively (Fig. 1B). The RGD motif present in the C-terminal domain of all mammalian IGFBP2 was also present in rtIGFBP2 (Fig. 1A).

IGFBP3

The sequence termed rtIGFBP3 (*DQ146966*) was 1142 bp in length, with an open reading frame coding for 283 aa. The encoded protein had an estimated molecular mass of 29 kDa and contained 20 cysteine residues (Fig. 1A). This aa sequence was 100% identical with the partial amino acid sequence of the previously characterized salmon IGFBP3 protein (Fig. 1C). Similar to mammalian IGFBP3, the rtIGFBP3 aa sequenced contained three *N*-glycosylation sites at positions 46, 112, and 258. rtIGFBP3 is clearly different from rtIGFBP2 (Fig. 1C), and the two proteins only share 56% at the aa level. rtIGFBP3 displayed 54% and 39% identity with zebrafish and human IGFBP2, respectively, but only 27% with zebrafish and human IGFBP3 (Fig. 1B). Similar to IGFBP2, rtIGFBP3 protein exhibited an RGD motif in the C-terminal domain. The GCGCCXXC motif is not perfectly conserved in trout, and the corresponding sequence is ACGCCPVC. This difference was found in all clones sequenced.

IGFBP4

The nucleotide sequence of rtIGFBP4 (*DQ146967*) was 820 bp in length with an open reading frame of 141 aa (Fig. 1A). This sequence corresponded to a partial coding region, and



B

	rtBP1	rtBP2	rtBP3	rtBP4	rtP5	rtBP6	rtBP-rP1	huBP1	huBP2	huBP3	huBP4	huBP5	huBP6	huBP-rP1	zeBP1	zeBP2	zeBP3	zeBP5
rtIGFBP1	100	30	29	26	32	23	18	36	28	27	34	33	28	17	57	29	27	29
rtIGFBP2	30	100	56	28	29	27	22	31	44	31	33	28	27	17	31	73	29	27
rtIGFBP3	29	56	100	27	30	24	19	30	39	27	32	28	28	29	31	54	27	29
rtIGFBP4	26	28	27	100	27	27	8	27	24	24	54	27	26	16	30	30	21	21
rtIGFBP5	32	29	30	27	100	26	21	28	27	35	36	54	30	20	29	27	36	75
rtIGFBP6	23	27	24	27	26	100	16	25	22	29	27	29	32	18	25	27	26	27
rtIGFBP-rP1	18	22	19	8	21	16	100	18	16	15	14	22	15	53	21	18	17	14

C

FIG. 1. A, Alignment of complete aa sequences of rtIGFBPs and IGFBP-rP1. GCGCCXXC, CWCV, and RGD motifs are *underlined*. *, Position of cysteine residues. B, Amino acid sequence identity between rtIGFBPs and cognate human IGFBP1 to -6, human IGFBP-rP1, and zebrafish -1, -2, -3, and 5. C, Comparison of partial N-terminal aa sequences of salmon IGFBP3 with rtIGFBP2–3 and zebrafish IGFBP2 aa sequences. The aa identical in at least two sequences are *shaded*.

we were not able to obtain the missing 5′ end by PCR screening of several cDNA libraries. The partial amino acid sequence had the highest identity with human IGFBP4 (54%) and less than 27% identity with other human IGFBPs (Fig. 2A). It contained a putative *N*-glycosylation site at position 13, as in mammalian IGFBP4, a thyroglobulin type I domain at position 82, and a CWCV motif at position 108 (Fig. 1A).

IGFBP5

The rtIGFBP5 sequence (*DQ206713*) was 2705 bp in length with an open reading frame coding for 270 aa (estimated size of 28 kDa; Fig. 1A) and was found to be more similar to human IGFBP5 (54% identity) than to other IGFBPs (<36%). Furthermore, this sequence exhibited 75% identity with cognate zebrafish IGFBP5 (*AAM51549*; Fig. 1B). In addition, rtIGFBP5 contained a putative nuclear localization signal at position 173–180 as well as 20 cysteine residues.

IGFBP6

The rtIGFBP6 sequence (*DQ190459*) was 2188 bp in length with an open reading frame of 199 aa (Figs. 1A and 2B). This is the smallest IGFBP, with an apparent molecular mass of 19 kDa and only 13 cysteine residues. Among all human IGFBPs, rtIGFBP6 exhibited the highest identity with human IGFBP6 (32%). Similar to mammalian IGFBP6, the GCGCCXXC motif is not conserved in rainbow trout, whereas another IGFBP-specific domain, the thyroglobulin type I domain, is present in the C-terminal domain (Figs. 1A and 2B). The rainbow trout sequence also contained a putative *N*-glycosylation site at position 6.

IGFBP-rP1

The seventh sequence (*DQ146965*) obtained had relatively low identity (<18%) with human IGFBPs (Fig. 1B). After comparison with human IGFBP-rP, it appeared that this se-

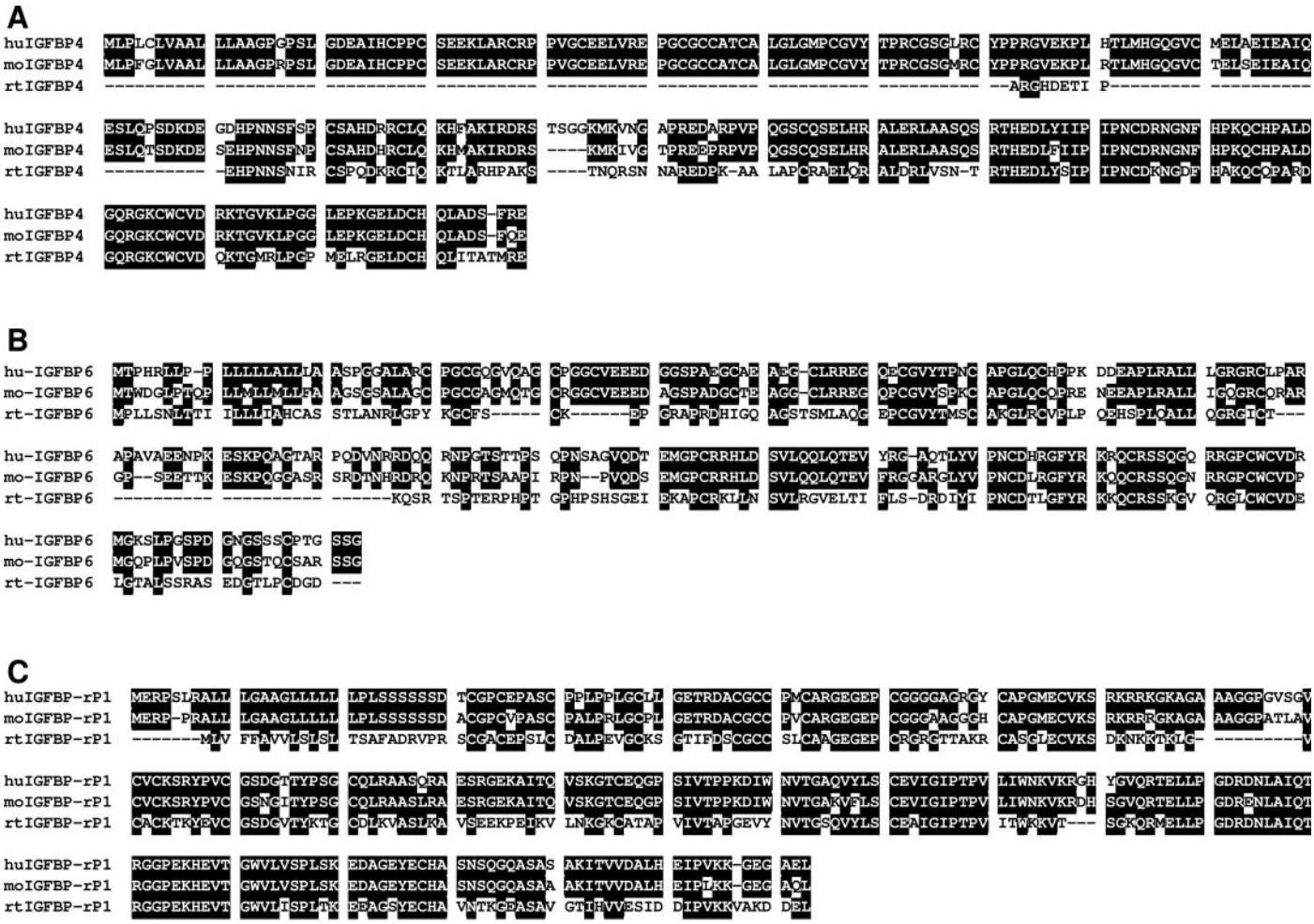


FIG. 2. Alignment of aa sequences of rtIGFBP4 (A), IGFBP6 (B), and IGFBP-rP1 (C) with cognate human (hu) and mouse (mo) IGFBP1, IGFBP6, and IGFBP-rP1. Corresponding GenBank accession numbers are presented in Fig. 3. Amino acids conserved between the three sequences are *shaded*.

quence had a high identity (53%) with IGFBP-rP1 (also termed IGFBP7), but it had less than 30% identity with other IGFBP-rPs. This sequence was 1217 bp in length with an open reading frame coding for a 263-aa protein exhibiting an estimated molecular mass of 26 kDa (Fig. 2C). As in mammals, the GCGCCXXC was not perfectly conserved and replaced by SCGCCSLC, but contained an Ig-like domain in the C-terminal part of the protein. It also contained a putative *N*-glycosylation site at position 154 as well as a nuclear localization signal at position 72.

Phylogenetic analysis

Representative vertebrate IGFBPs sequences, including all available fish sequences, were used to construct a phylogenic tree (Fig. 3). This analysis clearly showed that our sequences were located in six different clades corresponding to IGFBP1, -2, -4, -5, and -6 and IGFBP-rP1. All rtIGFBPs were grouped in a clade with very high bootstrap support (93%), except rtIGFBP3, which was located in the IGFBP2 clade, but with a very low bootstrap value (50%).

Tissue distribution of IGFBPs mRNA expression in rainbow trout

The tissue distribution patterns of IGFBPs were assessed by semiquantitative RT-PCR (Fig. 4). For IGFBP1, a PCR product at the expected size (725 bp) was observed only in liver. Despite 40 cycles of PCR, we were not able to detect IGFBP1 mRNA in ovarian tissue. In the brain, we obtained a higher size band (~1000 bp). This higher PCR product was constantly observed in several brain RT-PCRs (data not shown). IGFBP2 transcript was detected in all tissues except the eggs. IGFBP2 transcript was strongly detected in brain, liver, gill, muscle, trunk kidney, intestine, and stomach, whereas a comparatively lower expression was observed in skin, spleen, head kidney and ovary. Tissue distribution of IGFBP3 mRNA is clearly distinct from the IGFBP2 pattern. Indeed, IGFBP3 was only detected in liver, trunk kidney, skin, and gills, with a predominant expression in liver. IGFBP4 transcript was found at comparable levels in all tissues examined, except spleen and gills, in which no expression was detected. The presence of IGFBP5 mRNA was observed in most tissues, except gills, spleen, and eggs, and

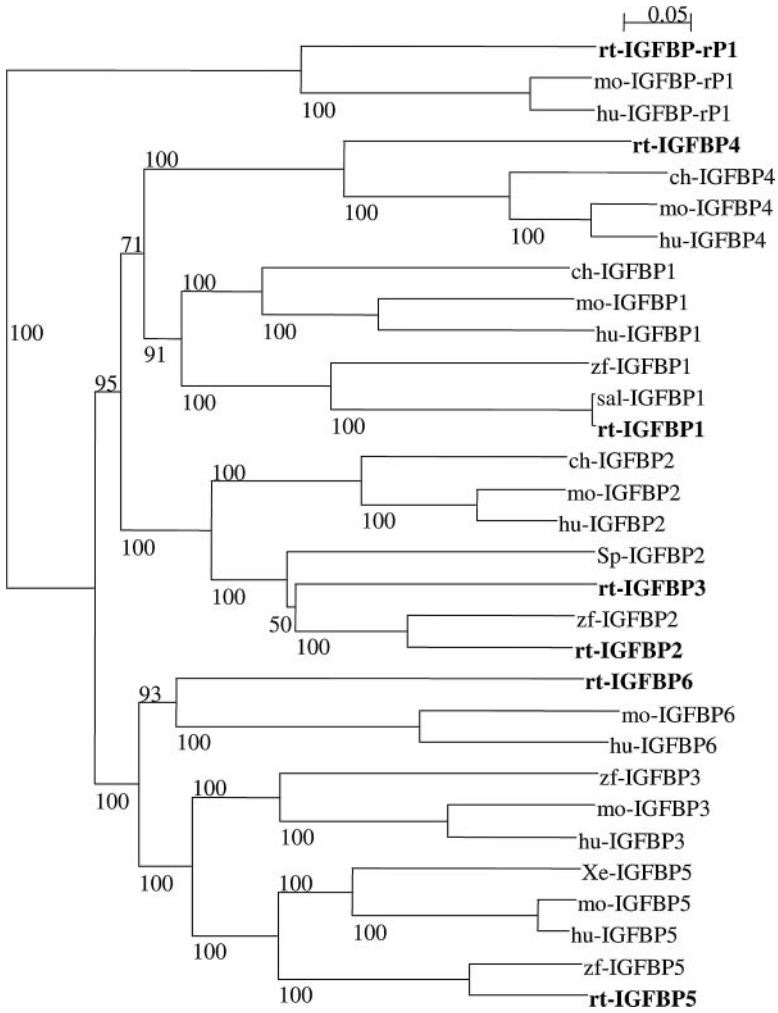


FIG. 3. Phylogenetic analysis of IGFBP aa sequences. Numbers on branches correspond to the percentage of times that the two clades branched as sisters (with 1000 runs). Branch length indicates proportionality to aa changes on the branch. The accession numbers for human (hu) IGFBPs are: IGFBP1 (*AAA52784*), IGFBP2 (*AAH71967*), IGFBP3 (*AAH64987*), IGFBP4 (*AAH16041*), IGFBP5 (*AAH11453*), IGFBP6 (*AAH05007*), and IGFBP-rP1 (*AAH66339*). The accession numbers for mouse (mo) IGFBPs are: IGFBP1 (*AAH13345*), IGFBP2 (*AAH12724*), IGFBP3 (*NP_032369*), IGFBP4 (*CAA53667*), IGFBP5 (*NP_034648*), IGFBP6 (*AAH12723*), and IGFBP-rP1 (*NP_032074*). The accession numbers for chicken (ch) IGFBPs are: IGFBP1 (*NP_001001294*), IGFBP2 (*NP_990690*), and IGFBP4 (*BAD83937*). The accession number for *Xenopus* (xe) IGFBP5 is *AAL12250*. The accession numbers for zebrafish (zf) IGFBPs are: IGFBP1 (*CAC44453*), IGFBP2 (*AAF23123*), IGFBP3 (*NP_991314*), and IGFBP5 (*AAM51549*). The accession number for salmon (sal) IGFBP1 is *AAV83995*. The accession number for sea bream (sp) IGFBP2 is *AAL57278*. The accession numbers for rtIGFBPs are: IGFBP1 (*ABA55020*), IGFBP2 (*ABA33956*), IGFBP3 (*ABA33954*), IGFBP4 (*ABA33955*), IGFBP5 (*ABA55021*), IGFBP6 (*ABA55019*), and IGFBP-rP1 (*ABA33953*).

weak expression was detected in the brain. IGFBP6 mRNA was expressed in all tissues analyzed, except the eggs, and the strongest expression was observed in spleen, gills, brain, and stomach. Among all IGFBPs, only IGFBP-rP1 transcript was expressed in all tissues examined, with the apparent highest levels observed in the brain and the lowest in the eggs.

Ovarian expression profiles

Among the seven IGFBP/IGFBP-rPs studied, only the IGFBP1 expression profile could not be monitored above the background level in the preovulatory ovary. The strongest mRNA abundance was observed for IGFBP4 and IGFBP-rP1, which exhibited 21.7 ± 1.3 cycle threshold (Ct; mean ± SD of the 18 ovarian samples) and 21.8 ± 0.7 Ct, respectively. Comparatively lower levels were observed for IGFBP5 (24.0 ± 0.9 Ct), IGFBP6 (26.0 ± 1.1 Ct), and IGFBP2 (26.3 ± 0.8 Ct), and the lowest levels were observed for IGFBP3 (31.5 ± 1.5 Ct). IGFBP2 and -4 did not exhibit any significant change in their mRNA abundance throughout the period. In contrast, IGFBP3 abundance exhibited a dramatic drop after vitellogenesis and remained low during oocyte maturation (Fig. 5). IGFBP5 mRNA abundance was significantly higher during the postvitellogenesis period than during late vitellogenesis (Fig. 5), whereas IGFBP6 mRNA abundance progressively increased during this period, although the rise was not significant until oocyte maturation (Fig. 5). Finally, IGFBP-rP1 mRNA levels were stable before meiosis resumption (late and post vitellogenesis) and exhibited a dramatic increase during oocyte maturation (Fig. 5).

Hormonal regulation

After a 60-h *in vitro* incubation, 100% germinal vesicle breakdown (GVBD) was observed for follicles incubated with gonadotropin at the highest concentration (188 ng/ml), whereas the lower concentration (23 ng/ml) did not induce GVBD. 17,20βP treatment induced only incomplete oocyte maturation, and traces of germinal vesicle remained. In contrast, no GVBD or any visible sign of oocyte maturation could be detected in control or E2-treated groups of follicles.

IGFBP2

IGFBP2 mRNA abundance monitored in intact follicles after a 20-h *in vitro* incubation was not affected by any hormonal treatment compared with the control group (Fig. 6A). In contrast, when follicles were deyolked after *in vitro* incubation, IGBP2 mRNA abundance was increased by gonadotropin (188 ng/ml), 17,20βP, and E2 treatments (Fig. 6B).

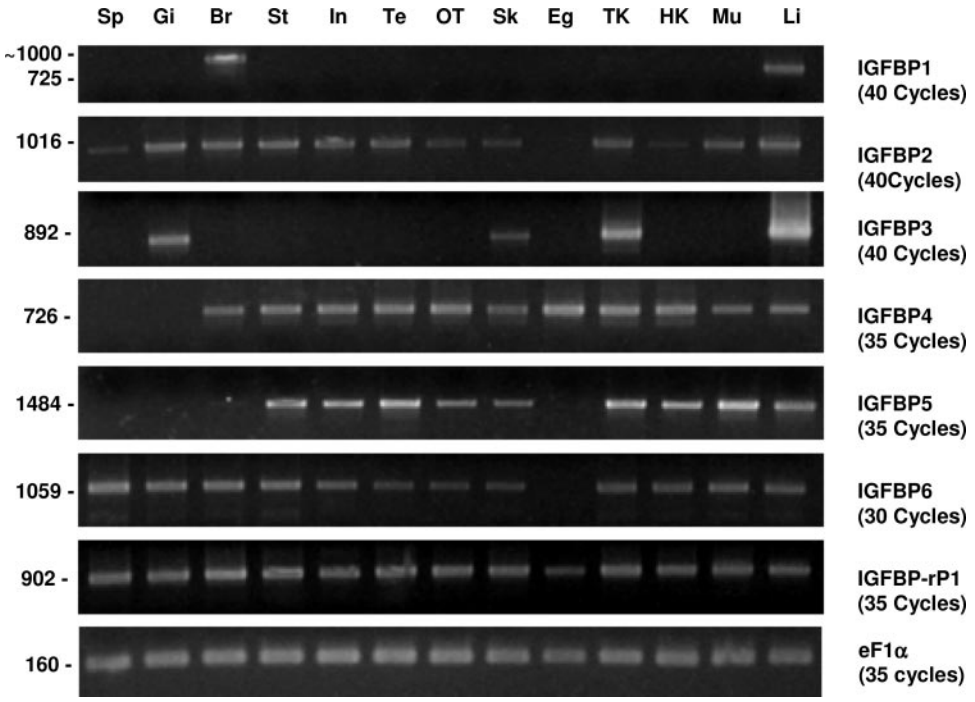


FIG. 4. Tissue distribution of rtIGFBPs mRNA. RT-PCR analysis was performed using total RNA originating from spleen (Sp), gill (Gi), brain (Br), stomac (St), intestine (In), testis (Te), deyolked ovarian tissue (Ot), skin (Sk), unfertilized eggs (Eg), trunk kidney (Tk), head kidney (Hk), muscle (μ), and liver (L). Amplification products after 30–40 cycles were analyzed on a 2% agarose gel and stained with ethidium bromide. Amplification of eF1α is shown below. The sizes of the PCR products are indicated on the *right*.

IGFBP3

IGFBP3 mRNA abundance monitored in intact follicles after a 20-h *in vitro* incubation was repressed by both gonadotropin and 17,20βP treatments (Fig. 6A). In contrast, E2 treatment resulted in a significantly higher IGBP3 mRNA abundance. When mRNA abundance was monitored in deyolked follicles, no significant difference was observed between hormonal treatment and control (Fig. 6B).

IGFBP4

IGFBP4 mRNA abundance monitored in intact follicles after a 20-h *in vitro* incubation was repressed by gonadotro-

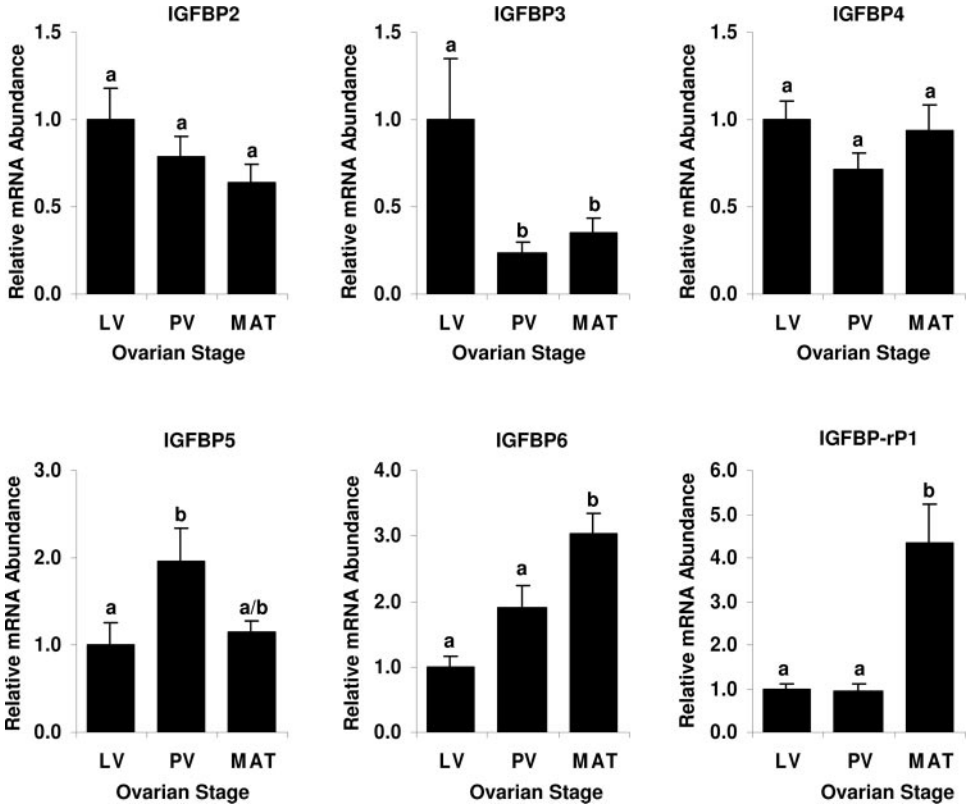
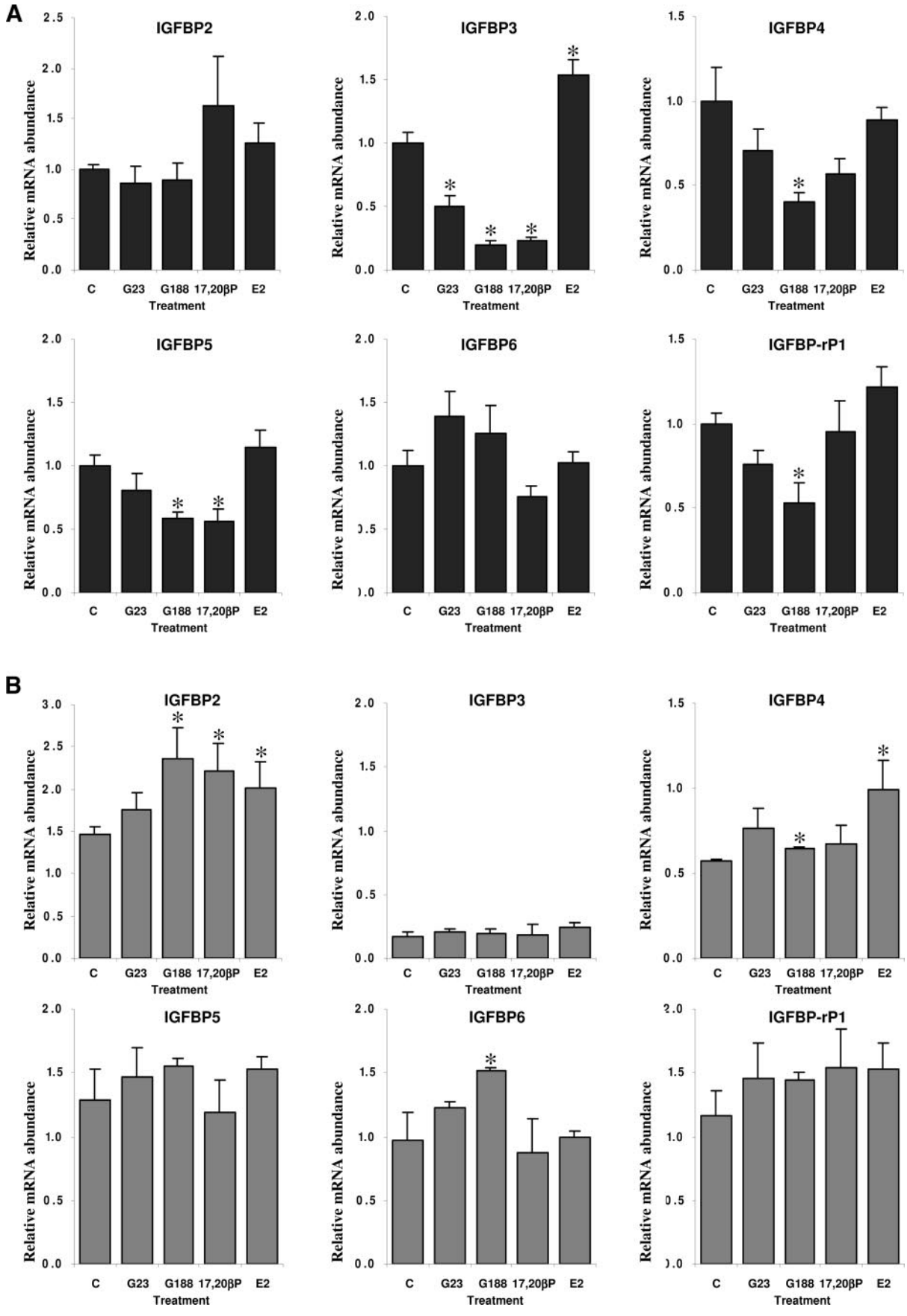


FIG. 5. Ovarian expression profiles of IGFBP1–6 and IGFBP-rP1 transcripts during rainbow trout oogenesis (mean ± SEM). Ovaries were sampled from separate females during late vitellogenesis (LV; n = 6), postvitellogenesis (PV; n = 6), and oocyte maturation (MAT; n = 6) stages. The transcript abundance of each transcript was determined by real-time PCR and normalized to the abundance of 18S. Abundance was arbitrarily set at 1 for the LV stage, and data are expressed as a percentage of the transcript abundance at this stage. *Bars* sharing the same letter(s) are not significantly different ($P < 0.05$).



pin treatment (188 ng/ml), whereas other treatments did not induce significant changes (Fig. 6A). Using RNA extracted after deyolking the follicles, a significantly higher abundance was observed after stimulation of the follicles by gonadotropins (188 ng/ml) and E2 (Fig. 6B).

IGFBP5

IGFBP5 mRNA abundance monitored in intact follicles after a 20-h *in vitro* incubation was repressed by gonadotropin (188 ng/ml) and 17,20βP treatments, whereas E2 treatment did not induce significant changes (Fig. 6A). In contrast, no significant change in IGFBP5 mRNA abundance was observed using deyolked follicles (Fig. 6B).

IGFBP6

IGFBP6 mRNA abundance monitored in intact follicles was not affected by hormonal treatments (Fig. 6A). In contrast, a significant up-regulation of IGFBP6 mRNA abundance by gonadotropin treatment was observed using deyolked follicles (Fig. 6B).

IGFBP-rP1

IGFBP-rP1 mRNA abundance monitored in intact follicles after a 20-h *in vitro* incubation was lowered by gonadotropin treatment (188 ng/ml), whereas other treatment did not induce significant changes (Fig. 6A). In contrast, the mRNA abundance of IGFBP-rP1 monitored after deyolking follicles was not affected by hormonal treatments (Fig. 6B).

Discussion

IGFBP1–6 and IGFBP-rP1 sequence characterization

In this study we report, for the first time within the same fish species the cloning and characterization of the six IGFBPs and one IGFBP-rP cDNAs. Although IGFBP1, -2, -3, and -5 have been described in zebrafish (10–12), tilapia (14), salmon (15), or seabream (13), it is the first time that IGFBP4 and IGFBP6 are described in a fish species. In addition, this study is also the first report of an IGFBP-rP in a nonmammalian species. The six high-affinity mammalian IGFBPs share a common domain organization: cysteine-rich N- and C-terminal domains and a central domain with no cysteine residues (1). This domain arrangement is well conserved within rtIGFBPs. Sequence alignment reveals that N- and C-terminal domains are well conserved across vertebrate species. In contrast, the central domain shows little sequence identity between trout and cognate human forms. Nearly all rainbow trout complete aa sequences contain 18–20 cysteine residues, as is the case in mammals (1). The unique exception is rtIGFBP6, which contains only 13 cysteine residues being rtIGFBP and the lowest number of cysteines, as observed in mammals (1). These cysteines are clustered in N- and the C-terminal domains. The high degree of conservation of N and C domains is not surprising, because recent data indicated that both N- and C-terminal domains are required for IGF binding (27). Within the N-terminal domain, the GCGCCXXC motif is well conserved among rtIGFBPs, except IGFBP3 and IGFBP6. Interestingly, this motif it is also absent from mammalian IGFBP6 (1). The significance of this motif is to date unknown, but it is probably not a determining factor in IGF binding. Indeed, it is absent in human IGFBP6, which exhibits a high affinity for IGFs, and it is present in some IGFBP-related proteins that are known to exhibit low affinity for IGFs (1). Moreover, this motif is present in several proteins unrelated to the IGFBP family and exhibiting no affinity for IGFs (1). Similar to mammals, the CWCV motif present in all mammalian high-affinity IGFBPs is also conserved in all rtIGFBPs (1–6), but not in IGFBP-rP1 (1). This well-conserved motif is clearly implicated in IGFs binding (27, 28). The presence of this CWCV motif in the partial IGFBP4 sequence along with the presence of a thyroglobulin-type I domain, the strong sequence identity with human IGFBP4 (54%), and the phylogenetic analysis demonstrate the identity of this sequence. Finally, rtIGFBP-rP1 contains an Ig-like domain in the C-terminal part of the protein that is specific for mammalian IGFBP-rP1 (1). However, the function of this domain remains unknown.

Despite the overall structural similarity, rtIGFBPs have diverged significantly from their mammalian orthologs. The overall sequence identities between trout and human IGFBPs ranged from 30–50%. Indeed, some dissimilarities can be observed. For instance, rtIGFBP1 does not have the RGD motif in the C-terminal domain, which is present in all mammalian counterparts. The RGD sequence has been demonstrated to be important for localization of proteins on the cell surface by interacting with the $\alpha_5\beta_1$ integrin (26). The lack of this motif has been already reported in salmon (15) and zebrafish (12), suggesting that acquisition of the RGD sequence to IGFBP1 might have occurred after the divergence of teleost from other vertebrates.

Our phylogenetic analysis revealed that the seven sequences of IGFBPs are grouped in only six clades because of the position of rtIGFBP3 in the IGFBP2 clade. This result shows higher sequence identity between rtIGFBP3 and rtIGFBP2 than between rtIGFBP3 and other IGFBP3. Indeed, rtIGFBP3 exhibit 27% sequence identity with zebrafish IGFBP3, but 54% with zebrafish IGFBP2. It is also noteworthy that rtIGFBP3 exhibits an RGD motifs, which is usually considered to be specific for IGFBP2 (and IGFBP1 in mammals) (1). A hypothesis to explain the presence of two sequences related to IGFBP2 could be a gene duplication, because salmonids are believed to have a comparatively recent tetraploid ancestry (29). However, the sequence identity between rtIGFBP3 and rtIGFBP2 is only 56% at the amino acid

FIG. 6. Hormonal regulation of IGFBP1 to -6 and IGFBP-rP1 mRNA abundance in intact (A) and deyolked (B) full-grown rainbow trout ovarian follicles after a 20-h *in vitro* incubation. For each hormonal treatment (C, Control; G23, G188, partially purified gonadotropin, 23 or 188 ng/ml; 17,20βP, 40 ng/ml; E2, 1 μg/ml), six replicates were performed. All replicates were performed using 25 follicles originating from the same ovary. Three replicates were used to extract RNA from intact follicles, and three replicates were deyolked before RNA extraction. The transcript abundance (mean ± SEM) of each transcript was determined by real-time PCR and normalized to the abundance of 18S. For each gene, mRNA abundance was arbitrarily set at 1 for the intact follicles in the control group, and the same y-axis scale was used in A and B to allow comparison of mRNA abundance between intact and deyolked follicles. *, Significantly different from control group ($P < 0.05$).

level. This homology is very low compared with the sequence identity observed between duplicated genes, which is usually greater than 80% (30–32). In addition, rtIGFBP3 branching in the phylogenic tree is too old to result from the recent duplication of the trout genome (29). For all these reasons, rtIGFBP3 cannot correspond to a duplicate gene as often found in salmonids. Furthermore, all other rtIGFBPs are grouped in their respective clade with a very high bootstrap value (>90%), whereas rtIGFBP3 is grouped in the IGFBP2 clade with a low bootstrap value (50%). This suggests that the rtIGFBP3 position in the IGFBP2 clade would not be fully reliable. Recently, Shimizu and colleagues (17) purified a 41-kDa IGFBP from serum of chinook salmon and could obtain a partial aa sequence (20 aa). Based on physiological responses, molecular mass, and glycosylation pattern, they concluded that this 41-kDa IGFBP was most similar to mammalian IGFBP3. Interestingly, this 20-aa sequence is 100% identical with the rtIGFBP3 identified here. In addition, we found *N*-glycosylation sites in rtIGFBP3, but not in rtIGFBP2, as in mammal's counterparts (33). Furthermore, after searching among the 350,000 salmon and trout ESTs available in public databases, no cDNA sequence putatively encoding for a protein with higher sequence similarity with IGFBP3s could be identified. Finally, a tissue expression study revealed that rtIGFBP3 is predominantly expressed in liver, which is one of the characteristics of IGFBP3 (34). For all these reasons, we propose to name this protein IGFBP3. However, it is still possible that another IGFBP3, exhibiting higher sequence homology with mammalian IGFBP3, exists in rainbow trout, but is absent from cDNA libraries due to a low expression level.

Tissue distribution

The tissue distribution pattern of rtIGFBPs was assessed by semiquantitative RT-PCR. rtIGFBP1 mRNA was only detected in liver, in agreement with the finding in zebrafish (12) and salmon (15) that liver is one of the main sites of IGFBP1 expression. Interestingly, a PCR product of unexpected size was consistently observed in brain RT products. However, the exact nature of this PCR product remains to be determined. IGFBP2 transcripts were found in many tissues, such as brain, gill, and liver, suggesting that IGFBP2 is synthesized locally in several rainbow trout tissues. This finding is in good agreement with previous observations showing that various fish tissues in culture produced a 31-kDA IGFBP that was considered to be the mammalian IGFBP2 homologue (35). IGFBP3 transcript was found in few tissues, with a predominant expression in liver, as observed in tilapia (14) and mammals (34). It is somewhat surprising that no signal was observed in the ovary lane even though we were able to monitor IGFBP3 expression in the preovulatory ovary by real-time PCR. However, the tissue distribution study was performed using deyolked ovarian tissue from a postvitellogenic female. Indeed, the lowest IGFBP3 levels were observed during this stage, and the hormonal regulation suggests that expression levels in deyolked tissue are much lower than those in intact tissue. Finally, IGFBP4 to -6 and IGFBP-rP1 were found to be expressed in a wide variety of tissues. This widespread expression is in good agreement with the mammalian tissue expression pattern (34) and strongly suggests that they may play a role in controlling IGF availability and activity in an autocrine and/or paracrine manner.

Ovarian expression and regulation

This expression study exclusively focused on mRNA levels of target proteins. For that reason, the mRNA expression profiles and hormonal regulations we report only suggest the involvement of corresponding genes in preovulatory ovarian physiology.

IGFBP1

IGFBP1 mRNA expression was not detected in preovulatory ovary or in unfertilized eggs. Our observations, therefore, suggest that IGFBP1 does not participate in preovulatory ovarian physiology.

IGFBP2

A real-time PCR gene expression survey previously revealed that IGFBP2 was expressed in the ovary before and during rainbow trout oocyte maturation (9). In seabream (*Sparus aurata*), IGFBP2 mRNA was detected in the cytoplasm of previtellogenic oocytes (36). In addition, immunostaining was observed in the cytoplasm of previtellogenic oocytes and in the follicular cells of yolk-granule-stage oocytes. In contrast, no signal was observed in oocytes at the perinucleolus stage (36). In the present study we observed IGFBP2 mRNA expression in rainbow trout preovulatory ovary. In addition, IGFBP2 mRNA abundance appeared stronger in deyolked follicles than in intact follicles. This observation is consistent with IGFBP2 mRNA expression in previtellogenic oocytes and follicular layers, as observed in seabream. In addition, IGFPB2 abundance is increased by gonadotropin, 17,20βP, and E2, when monitored in deyolked ovarian tissue, and the maximum up-regulation is observed after gonadotropin stimulation at a concentration inducing 100% oocyte maturation (assessed using GVBD occurrence). This increase, although significant, is limited and is not observed using intact ovarian tissue. In seabream, no significant increase in IGFBP2 mRNA abundance was observed after E2 stimulation at the reproductive stage (37). Together, our observations suggest that IGFBP2 mRNA abundance during the preovulatory period could increase under gonadotropic stimulation in follicular layers or extrafollicular tissue.

IGFBP3

In the present study we report ovarian expression of IGFBP3 mRNA. This observation is in agreement with the ovarian expression previously reported in zebrafish (14). However, these researchers report a predominant ovarian expression, which we did not observe in rainbow trout. During the preovulatory period, we observed a dramatic decrease in ovarian IGFBP3 mRNA abundance between late and postvitellogenic stages. During this period, follicles undergo follicular maturational competence acquisition, which can be defined as the follicle's ability to resume meiosis in response to a gonadotropic stimulation and subsequently

produce a developmentally competent oocyte (8). This period is also characterized by a drop in E2 (38) levels and an increase in LH (39) and 17,20βP (38) levels. Interestingly, in hormonally stimulated, intact ovarian follicles, IGFBP3 mRNA was strongly down-regulated by gonadotropin and 17,20βP, whereas a limited, but significant, up-regulation was observed after E2 stimulation. In contrast, a comparatively lower expression associated with no response to hormonal treatment was observed using deyolked ovarian tissue. This suggests that gonadotropin- and 17,20βP-induced IGFBP3 down-regulation and E2-induced up-regulation occur within the oocyte of the full-grown follicle. Together, our observations strongly suggest that IGFBP3 participates at the oocyte level in the process of follicular maturational competence acquisition.

IGFBP4

To our knowledge, IGFBP4 was never reported in any fish species. Therefore, no information is available on IGFBP4 expression in a fish ovary. In the present study we observed IGFBP4 expression in intact and deyolked follicles, suggesting expression in the full-grown oocyte and other cellular compartments of the ovary. No significant change in IGFBP4 mRNA abundance was observed during the preovulatory period. However, lower levels were observed in postvitellogenic females than in late vitellogenic fish. In addition, IGFBP4 was down-regulated by gonadotropin in intact follicles, but not in deyolked follicles. This indicates that IGFBP4 is down-regulated in the oocyte after gonadotropin stimulation. In contrast, IGFBP4 mRNA abundance measured after deyolking of the tissue was higher in gonadotropin- and E2-stimulated samples. However, the increase in IGFBP4 abundance after gonadotropic stimulation was extremely limited. It is therefore possible that a gonadotropin-dependent down-regulation of IGFBP4 mRNA occurs in the postvitellogenic oocyte. Finally, it is also noteworthy that IGFBP4 transcript is a maternally inherited transcript.

IGFBP5

IGFBP5 mRNA abundance exhibited a significant increase during the postvitellogenesis stage. *In vitro* hormonal stimulation of postvitellogenic follicles by gonadotropin and 17,20βP induced a significant down-regulation of the transcript. However, this could be observed using intact follicles, but not when follicles were deyolked before RNA extraction, suggesting that this down-regulation occurs in the full-grown oocyte.

IGFBP6

A progressive increase in IGFBP6 mRNA abundance was observed throughout the preovulatory period. However, the up-regulation of IGFBP6 transcript was not significant until oocyte maturation. No significant change in IGFBP6 mRNA abundance was induced in intact follicles by any of the hormonal treatments. In contrast, after deyolking the follicles, significant up-regulation was observed in gonadotropin-stimulated follicles, suggesting that this up-regulation occurs in follicular walls or adjacent extrafollicular tissue. Together, these observations suggest that gonadotropin-induced IGFBP6 up-regulation occurs during oocyte maturation in follicular walls or extrafollicular tissue.

IGFBP-rP1

No significant change in IGFBP-rP1 mRNA abundance was observed between late and postvitellogenesis periods. However, a sharp increase in IGFBP-rP1 mRNA abundance was observed at the time of oocyte maturation. Surprisingly, *in vitro* hormonal stimulation by gonadotropin induced a limited, but significant, down-regulation of the transcript. However, this could be observed using intact follicles, but not when follicles were deyolked before RNA extraction, suggesting that this down-regulation occurs in the full-grown oocyte. This, *in vivo* and *in vitro* data can appear somewhat inconsistent. However, *in vitro* data correspond to a 20-h incubation, whereas oocyte maturation occurs around 60 h. It is therefore possible that IGFBP-rP1 up-regulation occurs much later in the process of oocyte maturation, for example, immediately before ovulation. This would be consistent with the high levels of IGFBP-rP1 transcript detected in unfertilized eggs.

In conclusion, the present study reports, in addition to IGFBP1, -2, -3, and -5, the characterization of IGFBP4, IGFBP6, and IGFBP-rP1 for the first time in a fish species. We show that five IGFBPs and one IGFBP-rP are expressed in the trout ovary during follicular competence acquisition and oocyte maturation. Over this period, four transcripts exhibit a clear down- or up-regulation before or during oocyte maturation. In addition, all ovarian IGFBP mRNAs are regulated by gonadotropin at a concentration that induced 100% oocyte maturation, and four IGFBPs are also regulated by 17,20βP or E2. Together, these observations strongly suggest that IGFBPs/IGFBP-rP1 participate in regulating the action of the IGF system on final oocyte maturation in fish. Indeed, the present study shows that down-regulation of IGFBP3, -4, and -5 occurs in the oocyte under gonadotropic and 17,20βP (for IGFBP3 and -5) stimulation. In contrast, we observed an up-regulation of IGFBP2 and -6 in follicular layers or extrafollicular tissue under gonadotropic stimulation. It has been previously demonstrated that IGFs can modulate *in vitro* steroid production by follicular layers (5–7). In rainbow trout, it was demonstrated that both IGF-I and IGF-II transcripts are overexpressed in the ovary during oocyte maturation (9). It can therefore be hypothesized that IGFBP2 and -6 participate in the modulatory effect of IGFs on follicular steroid production. In contrast, IGFs can induce oocyte maturation in several fish species (3, 4) or increase oocyte responsiveness to the maturation-inducing steroid (3). This action is not blocked by transcription inhibitors, suggesting that it is independent of steroid production. From our observations, we can speculate that IGFBP3, -4, and -5 participate at the oocyte level in the direct action of IGFs on the oocyte. However, additional investigations are needed to unravel the precise contribution of each IGFBP protein to competence acquisition and oocyte maturation processes.

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B.B.K., J.-C.G., and J.B. have nothing to declare.

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