Insulin like growth factor-I and II binding and action on DNA synthesis in rainbow trout spermatogonia and spermatocytes

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Insulin-Like Growth Factor-I and -II Binding and Action on DNA Synthesis in Rainbow Trout Spermatogonia and Spermatocytes[1]

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ABSTRACT

Radio-labeled recombinant human insulin-like growth factor (^{125}I-rhIGF-I) bound specifically to total testicular cells and to spermatogonia plus primary spermatocytes (Go+CI) that had been prepared from trout testes at various maturation stages by centrifugal elutriation and then cleared of somatic cells by preculture in the presence of 2% Ultroser G. Binding sites showed high affinity (Ka = $0.5 \pm 0.2 \times 10^{10}$ M^{-1}) and low capacity (1.1 ± 0.8 fmol/10^6 testicular cells) for ^{125}I-IGF-I. (Gln^5, Ala^4, Tyr^{15}, Leu^{16})IGF-I ([QAYL]IGF-I) was equipotent to IGF-I in competing with ^{125}I-IGF-I for site occupancy on Go+CI. Insulin-like growth factor-II (IGF-II) was 3- to 10-fold less potent than IGF-I or (QAYL)IGF-I, while bovine insulin competed only at about 300-fold higher concentrations.

Go+CI were cultured for 3 days in the presence or absence of mammalian IGF-I, IGF-II, (QAYL)IGF-I, and salmon or bovine insulin. All these molecules stimulated the incorporation of [^{3}H]thymidine (added during the last 24 h in culture) by Go+CI in a dose-dependent manner. The mean ED_{50}, independent of testicular maturation stage, was 5.9 ± 4.9 ng/ml and 29.1 ± 15.8 ng/ml for IGF-I and IGF-II, respectively. (QAYL)IGF-I was as potent as IGF-I. Concentrations of salmon or bovine insulin 100- to 300-fold higher were required to produce effects similar to those of IGF-I. While recombinant human IGF binding protein (IGFBP-3) had no effect by itself on basal [^{3}H]thymidine incorporation, it inhibited the effect of IGF-I in a dose-dependent manner; however, it had no effect on the stimulation by (QAYL)IGF-I. Although combinations of low concentrations of IGF-I and IGF-II or salmon insulin had additive effects, combinations of maximum concentrations did not. We conclude that, in vitro, IGFs stimulate DNA synthesis of trout male germ cells by interacting directly with these cells through one IGF receptor.

INTRODUCTION

Spermatogenesis is a complex and unique developmental process. It can be divided into three phases: spermatogonial proliferation, meiosis, and spermiogenesis. The first phase starts with spermatogonial stem cells that divide and simultaneously maintain their number by renewal. Then, spermatogonial cell lineages derived from stem cells undergo successive mitotic divisions leading to several types of spermatogonia (Go). At least in mammals, the sequence of Go divisions is precisely regulated and strictly synchronized with the development of the other germ cells [1].

Although it is generally agreed that germ cell division and subsequent differentiation are dependent upon the presence of pituitary gonadotropins (FSH in mammals) and testosterone, there is now strong evidence that the effects of these hormones are indirect and are mediated by locally produced factors with a paracrine role. Several growth factors are known to be produced by different testicular cells (for review see [2, 3]). The physiological roles of most of these are still unknown. In particular, the triggers for the initiation of a spermatogenic wave and the factors regulating the proliferative activity of the different types of Go are not clear. In vivo and in vitro studies have suggested that testicular chalones, inhibin, activin, transforming growth factor β (TGFβ1), epidermal growth factor, interleukin-1α, and insulin-like growth factor-I and -II (IGF-I, -II) could be involved (for review see [2, 3]).

Among these factors, only IGFs [4], TGFβ1 [5], and activin [5, 6] have been shown to interact effectively with the cellular components of the seminiferous tubules. Except in the case of activin, which has been shown to bind to mitotic germ cells [6], the intratubular target cells for the other factors are not known. This is a consequence mainly of the lack of suitable in vitro models for culturing mammalian Go and spermatocytes (CI) in the absence of Sertoli cells—models that would allow checking for a direct action of growth factors on these germ cells. Although IGFs are presumed to be paracrine factors produced by Sertoli cells [7–9], conclusive evidence of a direct action of these factors on spermatogonia is still lacking. In addition, no IGF-I immunoreactivity has been detected in rat Go [7–10].

In most of the mammals studied, spermatogenesis is a continual process and the spermatogonial stem cells divide in a cyclic manner but continuously throughout the year. In teleosts that breed seasonally, such as salmonids, Go proliferation takes place only during a certain period of the year. As a consequence, from the onset of spermatogenesis to the end of the reproductive cycle (regression phase), the histological and cytological events throughout the testis tend to be synchronized. This situation offers technical advantages for addressing still-unresolved questions dealing with the control of spermatogenesis. In most of the teleosts, few data dealing with the number of Go types and generations and with the scheme of the renewal of stem cells are available. In the trout, type A Go are permanent germ cells in

the testis. In gonads undergoing spermatogenesis (maturation stages II to VI), they divide to provide type B Go. These two types of Go proliferate for about 6 mo. During the breeding season (maturation stages VII and VIII) and then the regression phase (maturation stage IX), Go cease multiplying and only quiescent type A Go are present [11].

Although such knowledge could be interesting from several points of view, very little information deals with the endocrine control of Go proliferation in fish, i.e., the factors that trigger the initiation of spermatogenesis. Experiments carried out in vivo or with testis fragments have suggested that gonadotropins such as maturational piscine gonadotropin (GtH II) and hCG, growth hormone (GH), and androgens (testosterone and 11-ketotestosterone) could participate in this control [12–14]. Until recently, it was not known 1) whether endocrine factors interact with Go and 2) which paracrine factors could act on Go to directly control their proliferative activity. Recently [15], we have observed that salmon GtH II, trout GH, and three testicular steroids do not change the in vitro proliferation of trout spermatogonia plus primary spermatocytes (Go+CI) uncontaminated by somatic cells. Conversely, preliminary data have suggested that recombinant human IGF-I (rhIGF-I) should be active on these germ cells. In dogfish, IGF-I stimulates [³H]thymidine incorporation by spermatocysts that contain germ cells and Sertoli cells [16].

We recently developed a germ cell culture system for trout testes obtained at various maturation stages [15]. In the present study, we used this system and binding techniques 1) to investigate whether IGFs were able to interact with specific binding sites on trout testicular cells and Go+CI and 2) to examine the effects of human IGF-I and -II, of an IGF analog with a reduced affinity for the IGF binding proteins (IGFBP), and of salmon and bovine insulin on DNA synthesis by Go+CI cultured for 3 days in the absence of Sertoli cells. Because neither native nor recombinant teleost IGFs were available at the time of this study, we have not compared the biological activities of human and salmonid peptides. However, the cDNAs of these peptides are highly homologous in their ligand binding domains [17, 18], and it has been observed recently that human and salmonid recombinant peptides are equally potent in an in vitro assay using cartilage of salmon [19]. We demonstrate for the first time in a vertebrate the presence of IGF-I-specific binding sites on testicular germ cells and a direct mitogenic effect of IGF-I and IGF-II on these cells.

MATERIALS AND METHODS

Materials

Collagenase (A, from *C. histolyticum*), mitomycin C, and hydroxyurea were from Boehringer-Mannheim (Meylan, France). Percoll was from Pharmacia (St. Quentin en Yvelines). Ultroser G (5-fold concentrated fetal calf serum substitute) was from IBF-Sepracor (Villeneuve-la-Garenne,

France). Hexadecyltrimethylammonium bromide (HTAB) was from Eastman Kodak (Rochester, NY). Deoxyribonuclease (DNase), α-tocopherol, retinyl acetate, ascorbic acid, antibiotics, culture media, and bovine insulin were from Sigma Chemical Co. (St. Louis, MO). Insta-Fluor was from Packard Instrument Co. (Groningen, The Netherlands). (Methyl ³H)thymidine (³H-Tdr; code TRK300, specific activity 925 GBq/mmol) and 5-bromo-2′-deoxyuridine (BrdU; cell proliferation kit II) were provided by Amersham (Little Ghalfont, England). Recombinant hIGF-I and hIGF-II were a gift of Ciba-Geigy (Summit, NJ). Non-glycosylated recombinant human IGFBP-3 was generously provided by C.A. Maack (Celtrix Pharmaceuticals Inc., Santa Clara, CA). Recombinant (Gln³, Ala⁴, Tyr¹⁵, Leu¹⁶)IGF-I ([QAYL]IGF-I, an analog with a reduced affinity for all IGFBPs [20]) was generously provided by M.A. Cascieri (Merck, Sharp & Dohme, Rahway, NJ). Salmon insulin was kindly donated by E. Plisetskaya (University of Washington, Seattle, WA).

Preparation and Culture of Cell Suspensions

Two-year-old male rainbow trout were used. They were kept in recycled freshwater at 12–15°C under natural photoperiod for at least 2 mo before being used. Fish were anesthetized with .3% phenoxyethanol and then killed by a blow to the head.

All operations were carried out in sterile conditions at 12–13°C. The techniques used to prepare and culture populations of germ cells have been described in detail elsewhere [15, 21, 22]. In brief, 2–6 testes were removed from at least two males at the same maturation stage, which was determined according to Billard and Escaffre [23]. Testes were then perfused for 5 h with a 0.8 mg/ml collagenase solution in culture medium with Mg^{2+} but not Ca^{2+}. The testes were cut into several pieces, resuspended in 100–150 ml modified L-15 Leibovitz medium [21] plus 1% BSA and 2% Ultroser G, and then left overnight with light shaking. The suspension ("total testicular cells") was then filtered on a 150-μm nylon filter.

When spermatozoa were present in significant numbers in the testicular cell suspension, most of them were removed by centrifugation (500 × g for 30 min and then 100 × g for 30 min) in an isotonic Percoll solution (mean density equal to 1.065 g/ml [21]). The floating cell layers ("testicular cells") were collected and then diluted six times with L-15 medium and cells were pelleted by centrifugation (50 × g, 10 min).

For IGF binding studies, besides the "total testicular cells," two other subpopulations were obtained as follows. After 1.5 days of culture in which the somatic cells were allowed to adhere to the plastic, the germ cell-enriched nonadhered cells were pelleted at 50 × g for 10 min while the attached cells were washed to remove the unbound germ cells. In addition, the upper layer of the pellet obtained after centrifugation in Percoll, containing only spermatozoa ("testic-
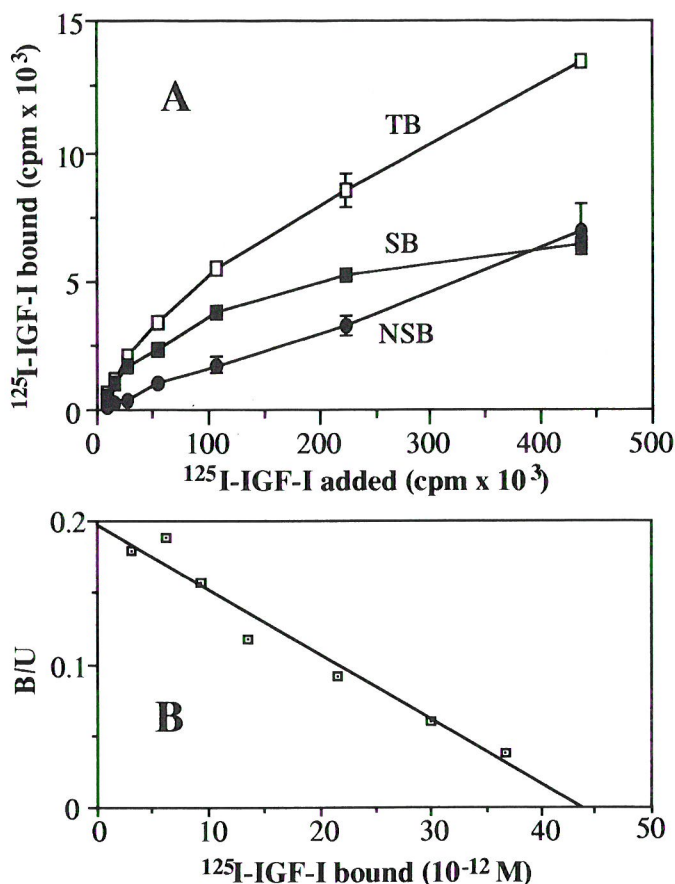
FIG. 1. A) Saturation curve of IGF-I binding to total testicular cells prepared in the absence of Ultroser. Cells (10^7 per assay tube) were incubated with increasing concentrations of ^{125}I-IGF-I (4–440 × 10^3 cpm/tube), in the absence (TB) or presence (NSB) of 100- to 1000-fold excess of unlabeled IGF-I. SB is the difference between TB and NSB. Mean ± SD (n = 3). B) Scatchard analysis of the data obtained from A. The ratio of bound to free (B/U) hormone is plotted against the specifically bound hormone concentration in the incubate. The plot results in a linear relationship, with a slope = Ka (affinity constant of the binding site).

ular spermatozoa"), was washed twice with culture medium.

Populations enriched in Go and CI were routinely prepared by centrifugal elutriation using a Beckman elutriator rotor (JE-5.0; Beckman Instr., Palo Alto, CA) with a standard 4-ml chamber. One or two elutriations were run: the elution medium was 0.5% BSA in L-15 medium plus 60 μg DNase/ml, temperature setting was 8°C, rotor speed was 2000 rpm. A maximum of 28 × 10^8 round testis cells (elongating and elongated spermatids and spermatozoa were not counted) suspended in 100 ml cold elution medium were loaded in the elutriation chamber at 7.8 ml/min; then 100 additional ml was collected at the same flow rate and 100 ml was collected at 9.5 ml/min. Two 150-ml fractions were collected, at 13 ml/min (Fr. I) and 19 ml/min (Fr. II). Cells present in these two fractions were pelleted and then washed once in L-15 medium by centrifugation (50 × g, 10 min). They were resuspended in L-15 medium plus 2% Ultroser

G (20 × 10^6 or fewer round cells/ml); they were then seeded in Nunc (Roskilde, Denmark) 80-cm^2 culture flasks (15 ml/flask) and left, routinely overnight, without shaking, at 12–13°C (preculture step).

Germ cells that had not adhered to the plastic during the preculture step were collected, centrifuged (50 × g, 5 min), washed twice in L-15 medium, and then counted. Routinely, between 0.4 and 2.5 × 10^6 Go+CI suspended in 400 μl L-15 medium were seeded per well in Nunc plastic 24-well plates. These numbers were chosen after preliminary experiments showing that, under our cell culture conditions, 1) ^{3}H-Tdr incorporation by 10^3 Go+CI remained constant when the number of Go+CI per well was lower than or equal to 2.5 × 10^6; 2) the ED_{50} for IGF-I was constant when at least 0.4 × 10^6 Go+CI were present per well; and 3) tritium self-absorption did not vary up to 3 × 10^6 Go+CI per well.

Two hours after seeding, molecules to be tested were added at various concentrations in quadruplicate wells. Routinely, cultures were kept for 3 days, under air, without shaking, at 12–13°C.

IGF-I Binding Studies

IGF-I was labeled with ^{125}I by the chloramine T method as modified by Martal [24] (specific activity 60 to 75 μCi/μg). In these experiments, maximum binding activity of ^{125}I-IGF-I, measured on increasing amounts of human placenta membrane, was 37% or 27% depending on the batch and the age of tracer. Scatchard plot analyses of the saturation data were performed with values for bound to free (B/U) hormone corrected for maximum binding activity of the tracer.

During all steps, the solutions and cells were kept at 0–2°C. Cultured cells were gently collected with a rubber policeman, pooled, and washed for 6 h in fresh culture medium before binding assay. Binding studies were performed in culture medium + 0.3% BSA (total volume: 250 μl). Aliquots (100 μl) of cell suspension (4–16 × 10^6 cells) were incubated in glass tubes with increasing concentrations of ^{125}I-IGF-I (0.4–440 × 10^3 cpm/tube; 4.4 pg/tube to 4.4 ng/tube; saturation experiments), in the absence (total binding: TB) or presence (nonspecific binding: NSB) of 100- to 1000-fold excess of unlabeled IGF-I. In competition experiments, cells were incubated with a fixed amount of tracer (20–30 × 10^3 cpm) and increasing concentrations of unlabeled hormones: 0.3–300 ng/ml for IGF-I and analogs, 10–10 000 ng/ml for bovine insulin. A low incubation temperature (0–2°C) was used to limit tracer or binding site degradation and to avoid internalization of ^{125}I-IGF-I after binding to the plasma membrane of living cultured cells [25, 26]. Under these conditions, 12–24 h of incubation under gentle shaking resulted in equilibrium. Incubation was interrupted by two successive washes with 800 μl of ice-cold incubation medium, rapidly followed by centrifugation (250 × g, 10 min) and aspiration of the supernatant.
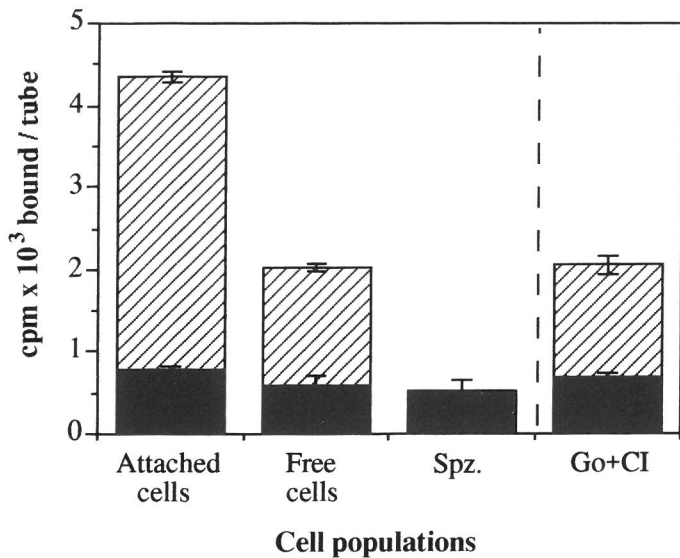
FIG. 2. IGF-I binding to different populations of testicular cells (prepared in the absence of Ultroser). The values shown are mean ± SD (n = 3) for ^{125}I-IGF-I TB (hatched + solid bars) and NSB (solid bars) in cpm per assay tube. Hatched bars correspond to SB. Cellular composition of the four populations obtained as described in *Materials and Methods* (spermatozoa that contaminate virtually all the populations were not counted, but they do not specifically bind ^{125}I-IGF-I): attached cells = 30% somatic cells, 70% germ cells (mainly Go); free cells = 9.5% somatic cells, 90.5% mixed germ cells; spermatozoa = ~100% pure; Go+CI = 0.4% somatic cells, 84% Go+CI, 15.6% other germ cells. The first three populations were tested after 1.5 days of culture and with 10^7 cells per assay tube. The Go+CI population was tested about 6 h after the end of the purification procedure and with 1.5×10^7 cells per assay tube. Total added ^{125}I-IGF-I: ~ 30×10^3 cpm.

Radioactivity bound to the pellet was measured in a Packard gamma counter (65% efficiency).

In binding experiments with blood serum, specificity was studied by incubating 25 μl of blood serum with ^{125}I-IGF-I in culture medium, in the absence or presence of unlabeled hormones, exactly as described for testicular cells. However, in this case, unbound hormone was removed at the end of incubation by adsorption on charcoal according to the procedure described by Niu and Le Bail [27] and modified as follows. Five hundred microliters of ice-cold culture medium containing 5% charcoal and 0.3% BSA was added to each tube. After a 15-min incubation the tubes were centrifuged ($4000 \times g$, 15 min), and the radioactivity remaining in the supernatant (bound ^{125}I-IGF-I) was counted.

[³H]Thymidine Incorporation

³H-Tdr was added (3.2 μCi/well, i.e., 78 ng/ml thymidine) for the last 24 h in culture. The cells were then cultured for an additional 4 h in fresh medium, without ³H-Tdr but containing 156 μg/ml thymidine. The nuclei were prepared with 0.4% HTAB plus 1 mM $CaCl_2$ [28]. They were counted, collected on GF/C Whatman (Clifton, NJ) filters, washed with 5% perchloric acid and then with ethanol, and dried. The incorporated radioactivity (cpm) was counted in
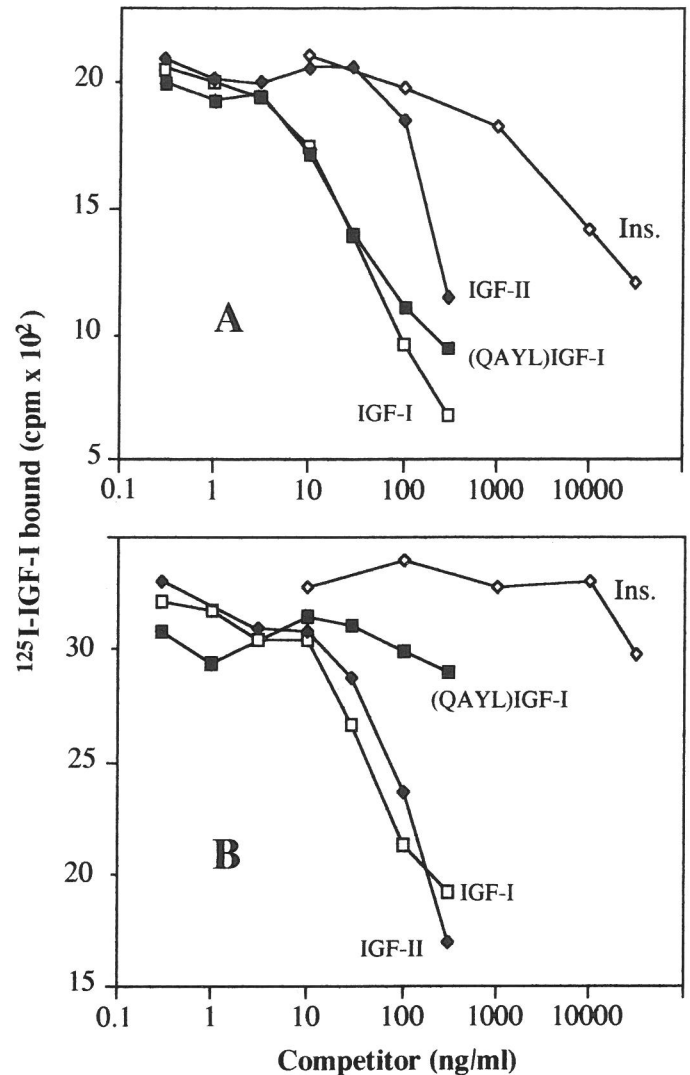

FIG. 3. Competitive binding assay between ^{125}I-IGF-I and unlabeled IGF peptides and (QAYL)IGF-I for binding to trout Go+CI ([A] Ultroser was present during the preculture step) and to trout serum proteins (B). The conditions were as described in *Materials and Methods*; 1.5×10^7 cells per assay tube were incubated with 37×10^3 cpm ^{125}I-IGF-I. Each of the ligands was tested at multiple concentrations. Each value is the mean of triplicate determinations of ^{125}I-IGF-I total binding. In the absence of competitor, B_0 in (A) was 1974 ± 75 cpm, i.e., = 5.4% of total added ^{125}I-IGF-I, and in (B) was 3176 ± 60 cpm, i.e., = 8.6% of total added ^{125}I-IGF-I.

the presence of Insta-Fluor in a Packard Tricarb counter. It was then ascribed to 10^3 Go+CI nuclei retrieved in the wells. Preliminary experiments had shown that when 3×10^6 Go+CI were seeded per well the amount of added ³H-Tdr was not limiting, even when the ³H-Tdr incorporation was stimulated by 4 times that of the control. Indeed, when 3.2 μCi ³H-Tdr was present per well for 24 h, 93.9 ± 3.4% (n = 4) of the added radioactivity was still present in the medium at the end of incubation. This amount was 91.4 ± 23 (n = 4) times higher than the amount incorporated in the nuclei (data dealing with controls and stimulation with IGF-I were gathered).
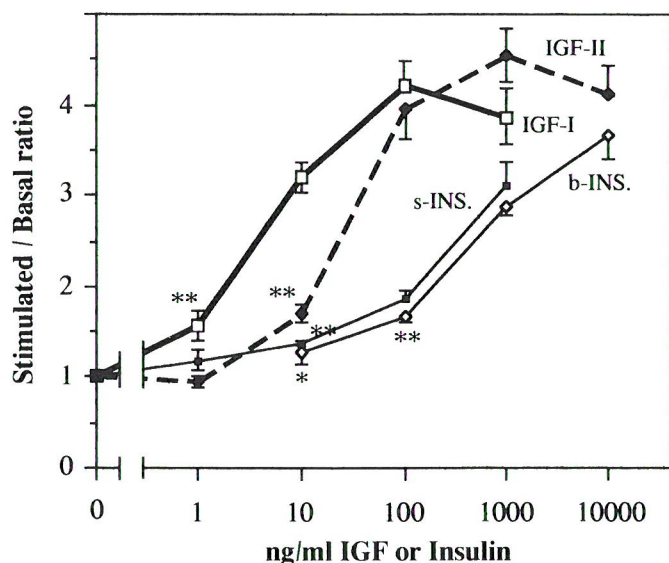
FIG. 4. Effect of increasing concentrations of rhIGF-I, rhIGF-II, salmon insulin, and bovine insulin on [3H]thymidine incorporation by Go+CI (2.4 × 10^6 per well) prepared from trout testes in early spermatogenesis. Cells were cultured for 3 days in the presence of one tested molecule; [3H]thymidine (8 μCi/ml) was present for the last day. Results are expressed as the stimulated:basal ratio; mean of four wells ± SD. Minimal stimulations significant with respect to basal 3H-Tdr incorporation: * p < 0.03, ** p < 0.015.

When Go+CI were cultured in the presence of 1.25 μg/ml mitomycin C and 0.4 mg/ml hydroxyurea (these molecules are assumed to block DNA synthesis), the 3H-Tdr incorporation routinely was between 4 and 8% of that measured in basal conditions. After BrdU labeling, it appeared that some scarce large Go escaped the blocking effect of mitomycin plus hydroxyurea.

When 2% formaldehyde was added simultaneously with 3H-Tdr, the mean background adsorption of 3H-Tdr was 0.9% of the basal incorporation.

BrdU Labeling

BrdU labeling was carried out according to the instructions of the manufacturer, with the modification that cells were incubated with 15 μM BrdU during the last 24 h in culture. Go+CI suspensions to be labeled were cultured either in suspension or on glass coverslips coated with poly-L-lysine [15]. Some samples of total testicular cells were also BrdU labeled.

Miscellaneous Techniques

Cells and nuclei (prepared with HTAB solution) were counted using a hemocytometer on a phase-contrast microscope. Germ cells and somatic cells, and their nuclei, were identified according to criteria given elsewhere [11, 22]. Testicular macrophages were also identified by an in vitro test (phagocytosis of carbon particles). Data were analyzed using the Mann-Whitney U test.

TABLE 1. Effects of rhIGF-I and rhIGF-II on the in vitro [3H]-thymidine incorporation by spermatogonia plus primary spermatocytes prepared from trout testes at various maturation stages. Values are mean ± SD.

Maturation stage[a]	IGF-I		IGF-II	
	ED$_{50}$ (ng/ml)	Maximum increase (St./B. ratio[b])	ED$_{50}$ (ng/ml)	Maximum increase (St./B. ratio[b])
III	3.3 ± 1.3 (n = 4)	3.9 ± 1.1 (n = 4)	22 (n = 1)	3.95 (n = 1)
III–IV	5.9 ± 4.7 (n = 6)	2.55 ± 0.5 (n = 6)	23.2 ± 14.8 (n = 2)	2.5 ± 0.9 (n = 2)
IV–VI	6.9 ± 6.4 (n = 7)	2.6 ± 0.7 (n = 7)	39 ± 16.2 (n = 2)	2 ± 0.2 (n = 2)
VIIIc–IX	10 (n = 1)	2.1 ± 0.2 (n = 2)		>1.95[c]
III–IX	5.9 ± 4.9 (n = 18)	2.8 ± 0.9 (n = 19)	29.1 ± 15.8 (n = 5)	2.5 ± 1.1 (n = 5)

[a]Stage III: beginning of spermatogenesis. Stages IV to VI: spermatogenesis. Stage VIIIc: end of spermiation. Stage IX: testis regression.
[b]St./B. ratio: mean stimulated:basal ratio.
[c]The highest tested IGF-II dose was 250 ng/ml.

RESULTS

Cellular Composition of the Go+CI Populations

Because cell separation by elutriation is based on cell size, secondary spermatocyte and spermatid symplasts (multinucleated cells [22]) with 2–4 nuclei were also present in Fr. I and II, which contained mainly Go and CI. When present, spermatozoa were not all removed by centrifugation in Percoll. During elutriation, they agglutinated in clusters and some (≤ 10% of the total cell number) were eluted in Fr. I and II. Thus 74 ± 13% (n = 11) of the cells seeded in culture (usually Fr. I and II were pooled after the preculture step) were Go and CI. Most of the other cells were round spermatids and secondary spermatocytes.
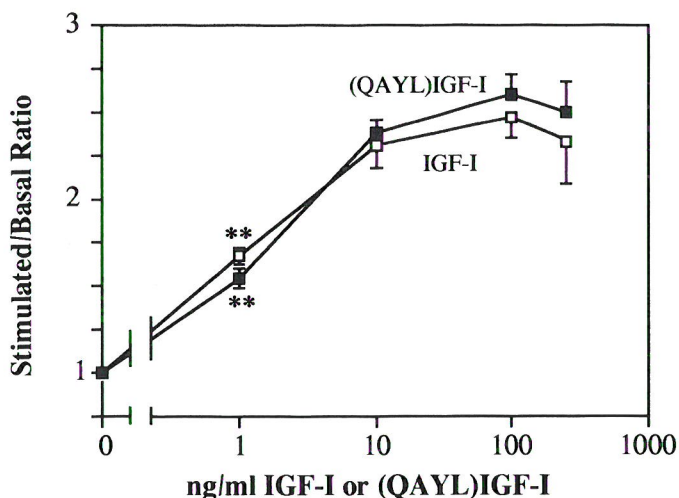

FIG. 5. Effect of increasing concentrations of rhIGF-I and (QAYL)IGF-I on [3H]thymidine incorporation by Go+CI (0.93 × 10^6 per well) prepared from testes in early spermatogenesis. For conditions of culture and of [3H]thymidine labeling and expression of results, see legend to Figure 4.
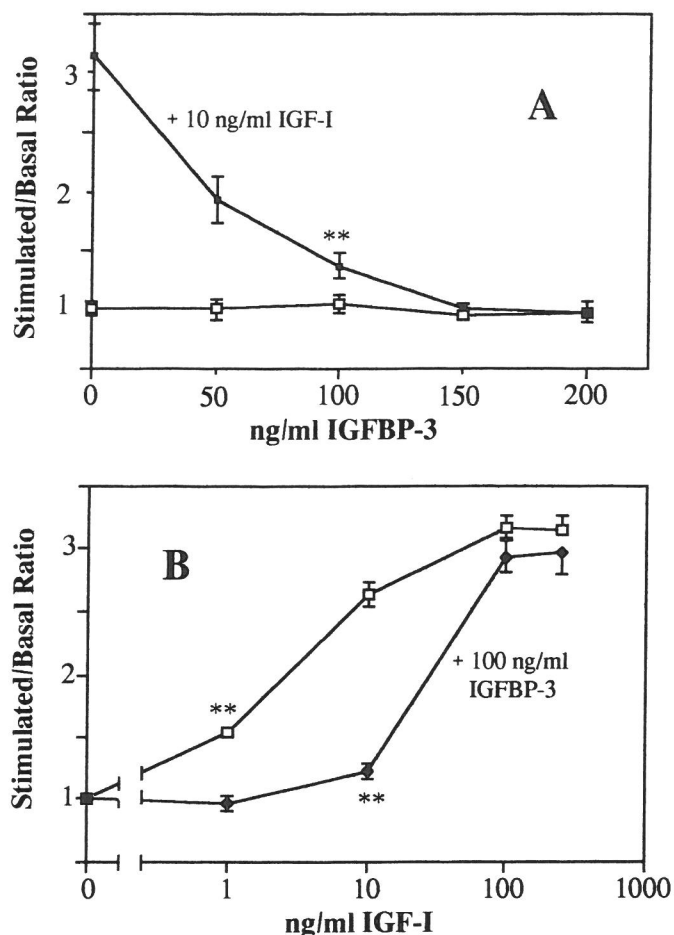
FIG. 7. Effect of increasing concentrations of (QAYL)IGF-I on [³H]thymidine incorporation by Go+CI (0.83 × 10⁶ per well) prepared from testes in early spermatogenesis. Cells were cultured in the presence or absence of 100 ng/ml IGFBP-3. Conditions of culture and of [³H]thymidine labeling and expression of results were as described for Figure 4.

FIG. 6. Effect of increasing concentrations of rhIGFBP-3 (A) and of rhIGF-I (B) on [³H]thymidine incorporation by Go+CI (1.8 × 10⁶ per well) prepared from testes in late spermatogenesis. A) Cells were cultured in the presence or absence of 10 ng/ml IGF-I. B) Cells cultured in the presence or absence of 100 ng/ml IGFBP-3. For conditions of culture and of [³H]thymidine labeling and expression of results, see legend to Figure 4.

ample, incubation of 4 to 8 × 10⁶ total cells with 21 000 cpm of tracer resulted in 10–16.5% specific binding (SB) and 4–8% NSB. When increasing concentrations of tracer were incubated with a fixed amount of cells, the binding sites appeared saturable and the Scatchard plot analysis of saturation experiments (Fig. 1) provided evidence for a single population of binding sites with high affinity for IGF-I (Ka ~ 0.5 ± 0.2 × 10¹⁰ M⁻¹, n = 3) and a limited number of sites (B_{max} = 1.1 ± 0.8 fmol/10⁶ cells).

Binding of ¹²⁵I-IGF-I was tested on four different populations of testicular cells: one enriched in round germ cells,

After elutriation, some testicular somatic cells (Sertoli cells, Leydig cells, myoid cells, and macrophages) were also present in Fr. I and II. Preculture for at least 6 h in the presence of 2% Ultroser G allowed all these cells to adhere to plastic. Careful observations of the culture plates after 3 days in culture confirmed that such cells had all been removed during the preculture step. Conversion of 17α-hydroxyprogesterone (0–3200 ng/ml) to 11-ketotestosterone in the presence of 400 ng/ml salmon gonadotropin (GtH II) by cells of the Go+CI population was not detectable, confirming the absence of Leydig cells [29]. Low numbers of erythrocytes (less than 1% of the total cell number) were usually present in Fr. II.

Binding of ¹²⁵I-IGF-I to Testicular Cells, Go+CI, and Blood IGFBP

Labeled IGF-I bound specifically to isolated cultured testicular cells as a function of the number of cells. For ex-
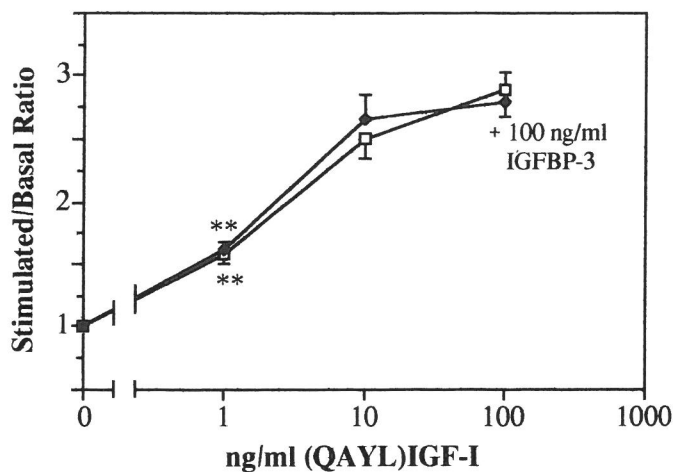

FIG. 8. Effect of rhIGF-I (hatched bars) and rhIGF-II (densely hatched bars and open bar) and of combinations of the two (cross-hatched bars) on [³H]thymidine incorporation by Go+CI (1.4 × 10⁶ per well) prepared from trout testes in mid-spermatogenesis. Four IGF-I concentrations (1, 3, 10, and 100 ng/ml) were combined with three IGF-II concentrations (1, 10, and 100 ng/ml, from left to right). Maximum increases were obtained for IGF-I with 100 ng/ml and for IGF-II with 1 μg/ml (open bar). Conditions of culture and of [³H]thymidine labeling and expression of results were as described for Figure 4. Bars with the same small letter are not significantly different.
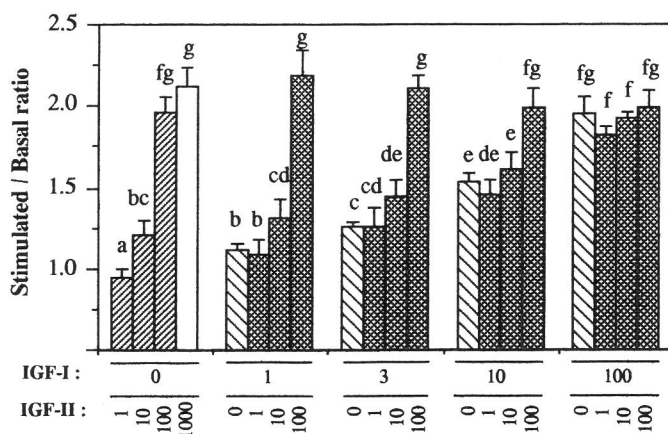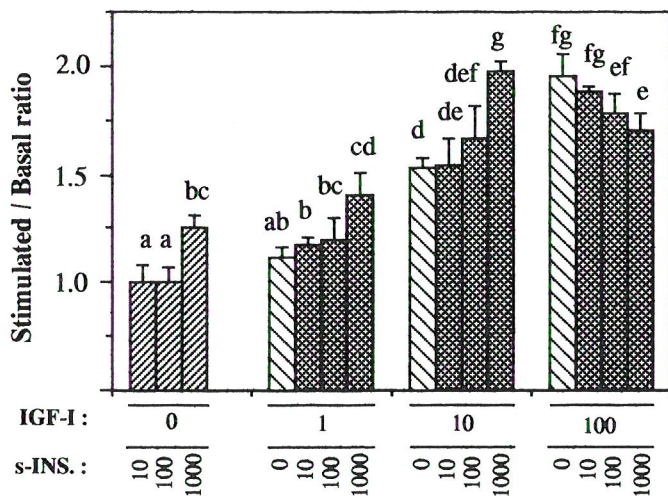
FIG. 9. Effect of rhIGF-I (hatched bars) and salmon insulin (densely hatched bars) and of combinations of the two (cross-hatched bars) on [^{3}H]thymidine incorporation by Go+CI (1.4 × 10^6 per well) prepared from trout testes in mid-spermatogenesis. Three IGF-I concentrations (1, 10, and 100 ng/ml) were combined with three salmon insulin concentrations (10, 100, and 1000 ng/ml, from left to right). Conditions of culture and of [^{3}H]thymidine labeling and expression of results were as described for Figure 4. Bars with the same small letter are not significantly different.

one enriched in somatic cells, one containing testicular spermatozoa, and one containing Go+CI purified by elutriation. As shown in Figure 2, specific binding was observed not only in the somatic cell-enriched fraction, but also in the germ cell-enriched fraction (although to a lesser extent) cultured for 3 days. No specific binding of IGF-I was observed on spermatozoa, while binding sites were present on Go+CI.

A competition study was carried out with purified Go+CI (Fig. 3A). ^{125}I-IGF-I binding was progressively inhibited in the presence of unlabeled IGF-I (3–300 ng/ml). As expected [20], (QAYL)IGF-I proved virtually equipotent to IGF-I. IGF-II was 3–10 times less potent. Insulin also competed with ^{125}I-IGF-I, but 50% inhibition of SB was obtained with a concentration of insulin about 300-fold higher than the concentration of IGF-I that induced the same inhibition.

A similar competition study conducted on trout serum (Fig. 3B) showed that IGF-I and IGF-II had similar apparent affinities for serum IGFBPs whereas (QAYL)IGF-I was a poor competitor and insulin was ineffective even at a concentration of 10 μg/ml.

Effects of IGF, Insulin, IGF Analogs, and IGFBP-3 on [^{3}H]Thymidine Incorporation

The BrdU-labeled cells were identified as Go and CI in the absence, as in the presence, of IGF-I. Usually no counts were carried out. However, in two experiments (maturation stages III and IV-V), the percentage of Go-CI that were labeled with BrdU was estimated to be equal to 20% and 17% in the absence of IGF-I and equal to 51% and 37% in its presence. When testicular cells were cultured on cover-

slips and BrdU labeled in the same conditions as the Go+CI populations, the majority of the labeled cells were Go and CI and only 0.9% were somatic cells.

After 3 days, ^{3}H-Tdr incorporation by the nuclei of Go+CI prepared from testes at various maturation stages was stimulated with either IGF-I or IGF-II, and this effect was dose-dependent (Fig. 4). A discrete but significant effect was observed as early as 1 day after addition of IGF-I (in this particular case, ^{3}H-Tdr was added every 12 h and the incubation was stopped 12 h later). With IGF-I, significant stimulation was obtained with as little as 1–3 ng/ml. The mean ED_{50} was equal to 5.9 ± 4.9 ng/ml (Table 1). Although this value displayed a tendency to increase throughout spermatogenesis, the increase was not statistically significant. The mean maximum stimulation was 2.8 times that of the control, and usually it was obtained with 100 ng/ml IGF-I. Usually, when a 3- to 4-fold stimulation of ^{3}H-Tdr incorporation was induced, the number of nuclei retrieved at the end of culture was about twice as high as in the unstimulated wells. (QAYL)IGF-I induced a dose-dependent stimulation of ^{3}H-Tdr incorporation identical to that induced by the same concentrations of IGF-I (Fig. 5).

Concentrations of salmon or bovine insulin about 100–300 times higher were required to produce effects similar to those observed with IGF-I. However, depending on the experiment (nine experiments), the lowest concentration of both insulins inducing a significant stimulation of ^{3}H-Tdr incorporation after 3 days in culture varied in the range of 10 ng/ml (Fig. 4) to 1 μg/ml (Fig. 9), without apparent relationship to the maturation stage of the testes.

Significant stimulation was obtained with 10 ng/ml IGF-II (Fig. 4). The mean ED_{50} was 29.1 ± 15.8 ng/ml (Table 1). The mean maximum stimulation, equal to 2.5-fold that of the control, was obtained with either 250 or 1000 ng/ml.

When IGFBP-3 was present alone (Fig. 6A), it had no effect on basal ^{3}H-Tdr incorporation. When it was added simultaneously with IGF-I, the stimulating effect of this growth factor was inhibited in a dose-dependent manner. The effect of 10 ng/ml IGF-I was completely inhibited by 150 ng/ml IGFBP-3. While 100 ng/ml IGFBP-3 inhibited the stimulating effect of 1 and 10 ng/ml IGF-I, this concentration did not significantly reduce the effect of 100 and 250 ng/ml IGF-I (Fig. 6B). In contrast, the stimulating effect of (QAYL)IGF-I was unchanged in the presence of IGFBP-3 (Fig. 7).

Combinations of low concentrations of IGF-I and IGF-II had additive effects, whereas combinations of maximum concentrations did not (Fig. 8). The maximum increases in ^{3}H-Tdr incorporation obtained with any combination of the two IGFs were never higher than the maximum increase obtained with the most potent IGF used alone (Fig. 8). Similar effects were obtained with combinations of IGF-I and salmon insulin (Fig. 9) and with combinations of IGF-I and bovine insulin (data not shown). However, the addition of

increasing concentrations of insulin to the most potent IGF-I concentration induced a progressively increasing inhibition of ^{3}H-Tdr incorporation.

DISCUSSION

In mammals, two types (1 and 2) of IGF receptors have been characterized in addition to the insulin receptor. The biological activity of IGFs is believed to be mediated largely through interaction with the type 1 receptor [30, 31] and also by cell surface-associated binding proteins [32, 33]. In the chicken, the type 2 receptor is absent [34]. In *Xenopus laevis*, it has been shown that insulin, IGF-I, and IGF-II exert their activity through the type 1 receptor [35]. In fish, only two studies have reported type 1 IGF receptors in fish liver and brain [36] and in ovaries [37]. Our observations demonstrated the presence of IGF-I-specific binding sites on trout testicular cells and male germ cells.

In competition binding assays, the observed affinity order (IGF-I = (QAYL)IGF-I > IGF-II >> insulin) is similar to that described for type 1 IGF receptor in mammals [20, 26, 38, 39] and in Xenopus [35]. Furthermore, this order is different from the affinity order observed in the present study with trout serum binding proteins (IGF-II ~ IGF-I >> (QAYL)IGF-I, insulin showing little or no affinity for the binding proteins), which is similar to that described for human IGFBPs [38]. These data suggest that, under our experimental conditions, IGF-I binding to the germ cells corresponded, at least in large part, to type 1 IGF receptors rather than to cell surface-associated binding proteins. While IGF receptors have been demonstrated on mammalian Sertoli cells and Leydig cells (for review see [2]), to our knowledge this is the first demonstration of functional IGF-I receptors on male germ cells in a vertebrate. Studies aimed at further characterizing IGF receptors in trout male germ cells will be undertaken.

The observed germ cell binding properties are in accordance with the relative biological potencies of the different peptides to stimulate ^{3}H-TdR incorporation in trout Go+CI. Duan and Hirano [40] have observed that IGF-II is about 50 times less potent than IGF-I in stimulating ^{35}S-sulphate uptake by eel cartilage. This growth factor is also less active (about 10 times less) than IGF-I in binding and stimulating the proliferation of trout male germ cells. (QAYL)IGF-I is an analog that has normal affinity for receptors but a 500–1000-fold reduced affinity for all IGFBP [20, 38]. Our observations confirm that it has the same affinity as IGF-I for the trout testicular IGF-I receptor and probably a low affinity for the binding proteins predominantly present in trout serum. As already observed in the case of rat A10 cells [20], (QAYL)IGF-I appeared to be equipotent to IGF-I in stimulating DNA synthesis in trout male germ cells. The stimulation of DNA synthesis induced by maximum concentrations of either IGF-I and IGF-II or IGF-I and insulin were not additive. These observations support

the hypothesis that the effect of the three peptides on DNA synthesis by Go+CI is mediated through the same IGF receptor. When present at high concentrations, insulin could restrain the access of the receptor to IGF when present at the most efficient concentration, thus resulting in a slight decrease of the stimulation of ^{3}H-Tdr incorporation.

Provided that elutriation is carried out at low temperature and DNase is present in the elution medium, this method, followed by a preculture step, allows mitotic germ cell populations to be obtained from trout testes throughout the reproductive cycle. These populations are devoid of somatic cells. In any case, BrdU labeling has confirmed that these latter cells had no significant proliferative activity during the first three days in culture; this is in concordance with previous data [22, 41]. Although germ cell populations contained mostly Go and CI (when these latter cells were present in the testes), in most cases they also contained variable amounts of secondary spermatocytes, spermatids, and spermatozoa. These cells are presumed not to synthesize DNA, and this was confirmed by BrdU labeling. Thus, they do not interfere directly with measurements of ^{3}H-Tdr incorporation by Go+CI. However, although to our knowledge no direct interaction between germ cells has been reported in mammals, it is unknown whether or not these postmeiotic germ cells could have a paracrine effect on the mitotic germ cell proliferation.

When spermatocytes are cultured in the absence of Sertoli cells, their survival is short (45–65% viability after 2 days [22]). In contrast, most Go cultured alone survive for several days. Because of this different survival capacity of the two cell types, cell numbers were not taken into consideration to check for the effect of IGFs on the proliferative activity of the mitotic germ cells. Data obtained with BrdU labeling on the one hand and in the presence of mitomycin + hydroxyurea on the other have shown that the ^{3}H-Tdr incorporation we measured represents true DNA synthesis. The increase in the number of Go+CI nuclei retrieved after an efficient IGF-I stimulation confirmed that this DNA synthesis occurred with proliferation of mitotic germ cells.

Our data confirm that mammalian IGF are active in teleosts [40, 42]. The present study demonstrates for the first time a direct mitogenic effect of both IGFs on the Go and CI in any vertebrate, including mammals. In situ, trout spermatogonia divide actively during the spermatogenetic phase and then are quiescent during subsequent stages. Although the ED_{50} for both IGFs seemed to increase throughout spermatogenesis (Table 1), it is not possible, because of a non-negligible interexperiment variability, to know whether the intrinsic sensitivity to IGFs ($1/ED_{50}$) of Go+CI proliferating in vivo (maturation stages III to VI) differs significantly from that of the Go+CI not proliferating (maturation stages VIII to IX). The presence of IGF-I and IGF-II in teleosts is now well established [18, 43, 44]. Moreover, both IGF-I and IGF-II mRNA have been observed in trout testis

extracts [45]. The effect of IGFs on Go+CI proliferation was demonstrated in vitro, and additional data are needed to confirm its in vivo occurrence. Nevertheless, our data suggest that, in the trout, IGFs participate in the regulation of spermatogonial proliferation and perhaps DNA duplication in spermatocytes by acting directly on these cells. In mammals, IGFs stimulate DNA synthesis in spermatogonia included in segments of seminiferous tubules [4], and in dogfish, IGF-I enhances ^{3}H-Tdr incorporation by spermatocysts [16]; but further studies would be necessary to elucidate whether IGFs interact directly with these cells. Indeed, IGF-I immunoreactivity has never been detected in rat spermatogonia, while it has been observed in spermatocytes [7, 9, 10].

IGFBPs that regulate the availability of free IGFs to the IGF receptors are important modulators of cellular responsiveness to IGF-I. IGFBP-3 has the capacity both to potentiate and to inhibit IGF-I action, depending upon the culture conditions [33]. Coincubation of IGF-I and IGFBP-3 resulted in dose-dependent inhibition of the IGF-I-stimulated proliferation of trout male germ cells. Conversely, in the same culture conditions, IGFBP-3 did not modify the effect of (QAYL)IGF-I. These observations are in accordance with other reports showing that IGFBP-3 inhibits IGF-I biological effects and especially DNA synthesis and cellular growth [33, 46] by sequestering the peptide and preventing receptor interaction.

In rat testis, IGF-I mRNA is expressed in Sertoli cells, Leydig cells, spermatogonia, and spermatocytes [9]. In addition, Leydig cells, at least in the boar, express a gene coding for a unique insulin-like factor (Ley I-L [47]). In the trout testis we know only that IGF-I mRNA and IGF-II mRNA are present [45, 48]; the cell types that could be involved in production of IGFs are not known, and the origin of IGF as well as the molecular species (IGF-I, IGF-II, or Ley I-L?) acting on spermatogonial proliferation remains to be determined. The observation that IGFBP-3 did not change the basal proliferation of Go+CI suggests that, at least in vitro, these cells would not produce IGF-I themselves. On the other hand, the observation that IGFBP-3 modulates IGF-I action makes this protein a candidate for participating in the regulatory pathways that, in vivo, allow precise control of germ cell division in trout testis. In the rat, IGFBP-3 is the predominant IGFBP produced in vitro by Sertoli cells, its abundance in the medium being influenced by IGF-I and cAMP [49].

In conclusion, our binding experiments directly show that it is possible for IGFs to act at the mitotic germ cell level. One direct action of IGFs on germ cells would be to stimulate their proliferation. The data obtained with hIGFBP-3 suggest that, in situ, IGFBPs possibly produced by Sertoli cells [49] could modulate this action.

ACKNOWLEDGMENTS

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