Expression of the Insulin Receptor in the Retina of the Goldfish

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PURPOSE. Insulin is a peptide growth factor that is active in most tissues, both during development and in adulthood. The action of insulin is through its specific membrane receptor. Previously retinal progenitors in the adult goldfish were shown to proliferate vigorously when exposed to insulin in vitro. The present study was undertaken to clone and characterize partial cDNAs that encode the goldfish’s insulin receptor (IR) and to establish the cellular pattern of expression of this gene in the retina.

METHODS. Standard methods were used for RNA isolation, reverse transcription–polymerase chain reaction, Northern blot analysis, and in situ hybridization.

RESULTS. Multiple clones were isolated that, based on sequence analysis, segregated into two groups, presumed to represent two genes that encode the IR. These clones were designated goldfish IR-1 (gfIR-1) and goldfish IR-2 (gfIR-2). Northern blot analysis showed that both genes are expressed in multiple tissues, including the retina. Both gfIR-1 and -2 give rise to a single, major transcript, but the sizes of the two transcripts are different. In situ hybridizations using digoxigenin-labeled riboprobes showed that gfIR-1 and -2 are expressed by all differentiated retinal neurons as well as neuronal progenitors in the circumferential germinal zone.

CONCLUSIONS. These data demonstrate that the IR is expressed in the retina of the goldfish, and, on the basis of the cellular pattern of expression, suggest that insulin may act both to regulate neurogenesis and influence the function of differentiated neurons. The cellular coexpression of the receptors for both insulin-like growth factor (IGF) 1 and insulin suggests that neurons and/or neuronal progenitors in the retina of the goldfish may contain hybrid IGF-1/insulin receptors. (Invest Ophthal Vis Sci. 2001;42:2125–2129)

The retina of teleost fish is a popular model for studying the development, plasticity, and regeneration of the central nervous system. The retina of the goldfish grows continually by both a balloon-like expansion and the addition of new neurons. New neurons, with the exception of rod photoreceptors, are generated from an annulus of retinal progenitors, known as the circumferential germinal zone (CGZ), that resides at the junction of the retina and iris. Rod photoreceptors are produced interstitially and once born are insinuated into the existing array of photoreceptors. In addition to this persistent, growth-associated neurogenesis, the retina will regenerate if injured.

In an attempt to identify molecules that regulate growth-related and injury-induced neurogenesis in fish, Boucher and Hitchcock tested numerous peptide growth factors and found that those structurally related to insulin (insulin, insulin-like growth factor [IGF] 1 and IGF-2) are potent mitogens of retinal progenitors within the CGZ. The proliferative response to these peptides was robust and dose dependent. The study described here was undertaken confirm that the mitogenic activity of insulin is via the insulin receptor (IR). To this end, partial cDNAs encoding the IR in the goldfish were cloned and the expression of the IR gene in the retina was characterized. Preliminary reports of this study have been published previously in abstract form.

MATERIALS AND METHODS

RNA Isolation, RT-PCR, and Subcloning

The handling and treatment of all animals used in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Goldfish (4–6 in. standard length) were dark adapted to aid in isolating the retinas and killed by exsanguination. The retinas were dissected from the globes and immediately submerged in TRIzol Reagent (Gibco BRL, Gaithersburg, MD). Retinas were homogenized, and total RNA was isolated using the single-phase, phenol/guanidinium method described by the manufacturer. Similar methods were used to isolate total RNA from brain, gill, heart, skeletal muscle, kidney, and liver. Precipitated RNA was resuspended in RNase-free water and used immediately for the reverse transcription–polymerase chain reaction (RT-PCR) or resuspended in deionized formamide and stored at −80°C (for Northern analysis).

First-strand cDNA synthesis was performed using the Superscript Preamplification System (Gibco BRL) according to the manufacturer’s instructions. The PCR was performed in a total volume of 50 µl containing 2 µl of the RT reaction, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTPs, and 2.5 U Taq DNA polymerase. Degenerate oligonucleotide primers, reported previously to amplify insulin receptor cDNAs from the gill cartilage of coho salmon (primers IR-3 and IR-10), were used at a concentration of 100 ng/reaction. The PCR reaction was allowed to run for 30 cycles (95°C for 30 seconds; 55°C for 2 minutes; 72°C for 2 minutes) followed by 72°C for 10 minutes.

PCR products were separated on a 1% agarose gel, and a single band at approximately 1800 bp was purified and subcloned using pGEM-T vector (Promega, Madison, WI). Plasmid DNA was purified from multiple bacterial colonies, and the inserts were completely sequenced on both strands with overlapping runs by the DNA Sequencing Core at the University of Michigan. Raw sequence data were assembled using the Sequencing Project Manager software (DNA Star; Lasergene, Madison, WI). Open reading frames were identified and translated, and the nucleotide and deduced amino acid sequences were compared with previously published sequences (GenBank Database).

Northern Analysis

Blots of total RNA from several tissues were probed for the Northern blot analysis. Twenty micrograms of RNA was loaded in each lane, separated electrophoretically in an agarose gel, transferred to Hy-
bond filters (Amersham Pharmacia Biotech, Amersham, United Kingdom) and photocross-linked (Stratalinker; Stratagene, La Jolla, CA). Blots were hybridized overnight at 42°C with random-primed,32P-labeled probes (13108 cpm; rediPrime II; Amersham Pharmacia Biotech) and washed the next day in 2× SS/0.5% SDS at room temperature for 30 minutes, followed by a wash in 0.1× SS/0.5% SDS at 55°C for 30 minutes. Hybridization signal was detected by exposing the blots to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA). Each blot was stripped with boiling 0.1× SS/0.5% SDS and reprobed.

Tissue Processing and In Situ Hybridization

Briefly, eyecups were fixed for 1 to 2 hours in fresh 4% paraformaldehyde, in 100 mM phosphate buffer, pH 7.2, and immersed overnight at 4°C in 20% sucrose in phosphate-buffered saline (PBS). The next day eyecups were infiltrated in a solution containing a 2:1 ratio of 20% sucrose/PBS and O.C.T. Compound (Tissue-Tek; Sakura Finetek, Torrance, CA), embedded in 100% O.C.T., and frozen. Retinas were sectioned at 10 μm using a cryostat and mounted on TESPA-coated slides (Sigma, St. Louis, MO).

FIGURE 1. Alignment of deduced amino acid sequences of IRs from goldfish, human, salmon, and turbot. Highlighted in bold are conserved tyrosine (Y) and cysteine (C) residues, the proteolytic cleavage site that separate the α- and β-subunits of the insulin receptor (RRRR), and the signature sequence identifying the intracellular kinase domain (GxGxxG21xK). The putative transmembrane domain is underlined. GenBank Accession numbers: gfIR-1, AF218355; gfIR-2, AF321225; human IR, NM 000208/NM 000207; salmon (sir-1), AF021040; turbot IR, AJ224994.

RESULTS

RT-PCR using retinal RNA and degenerate oligonucleotide primers9 amplified cDNAs with homology to vertebrate se-
sequences for both the IGFR-1 and the IRs. A description of the goldfish IGFR-1 receptor will be reported elsewhere [Otteson DC, Cirenza PF, Hitchcock PF, unpublished results]; see Ref. 11 for a preliminary report.) Comparison of the nucleotide sequences showed that clones encoding the goldfish IR were partial cDNAs, which segregated into two groups that were 67.9% identical (data not shown). Clones in the first group, designated gfIR-1, were 1740 bp in length; clones in the second group, designated gFIR-2, were 1752 bp in length. The deduced amino acid sequences (Fig. 1) show that both gFIR-1 and gFIR-2 encode a portion of the extracellular α-domain, the transmembrane domain, and a portion of the intracellular β-domain. In addition, both clones contain sequences common to all receptor tyrosine kinases, including conserved cysteine (C) and tyrosine (Y) residues, the tetra basic proteolytic cleavage site (RRRR/RQRR), and the tyrosine kinase signature sequence (GxGxxG1xK).12 gFIR-1 and gFIR-2 share similar levels of identity when compared with equivalent sequences of IRs from humans and fish (salmon and turbot; Table 1, Fig. 1). A pairwise comparison of amino acid sequences between gFIR-1 and -2 and cDNAs for the two goldfish IGFR-1 receptors showed that the goldfish IR was more similar to IR homologues from other animals than to goldfish IGFR-1 (Table 1), consistent with the interpretation that gFIR-1 and gFIR-2 are distinct from the cDNAs encoding the IGFR-1 receptor. Further, comparisons between gFIR-1 and -2 showed that they are only 69.7% identical (Fig. 1 and Table 1). We interpret this difference in identity, at both the nucleotide and amino acid levels, to indicate that gFIR-1 and -2 transcripts represent two, nonallelic genes encoding the IR.

The tissue distribution of IR transcripts was determined in Northern blots that were sequentially probed with gFIR-1 (Fig. 2A) and gFIR-2 (Fig. 2B). Probes synthesized from gFIR-1 hybridized with a single transcript at approximately 11 kb. The expression of level the 11-kb transcript is highest in retina, lowest in muscle, and present in brain, gill, heart, kidney, and liver. In contrast, probes synthesized from gFIR-2 hybridized with a major transcript at approximately 7 kb. Similar to the high-molecular-weight transcript of gFIR-1, gFIR-2 expression is highest in the retina, lowest is in skeletal muscle, and present in brain, gill, heart, kidney, and liver.

Figure 3 illustrates the cellular expression of gFIR-1 as revealed by in situ hybridization. Consistent with the high levels of expression of IR transcripts observed by Northern blot analysis, the IR appears to be expressed by most, if not all differentiated neurons (Fig. 3A). Qualitatively, the expression appears to be highest for ganglion cells and lower, but uniform, among neurons in the inner and outer nuclear layers. gFIR-1 is also expressed by the retinal progenitors within the CGZ and cells of the adjacent unpigmented iris epithelium (Fig. 3B). In situ hybridization with gFIR-2 yielded a pattern of hybridization similar to that for gFIR-1 (data not shown).

### Discussion

Insulin is an evolutionarily ancient polypeptide that is the defining member of a family of related peptides that regulate proliferation and cellular metabolism, differentiation, and growth.13–16,24,25 The action of insulin is mediated by a high-affinity, receptor-tyrosine kinase that shares structural features with all receptor-tyrosine kinases12 and is most similar to the receptor for IGFR-1.17 Although principally studied in mammalian systems, IRs are expressed in various fish tissues,18 including the brain and retina (see Results). Unlike mammals (and birds), however, insulin is not synthesized in the teleost brain, suggesting that insulin acting in the avascular retina of goldfish must be actively transported from the circulation.

Sequence comparisons of the partial cDNAs encoding the IRs in goldfish revealed that clones segregated into two groups that at the nucleotide level were only 67.9% identical. This relatively low degree of similarity cannot be attributed to amplification or sequencing errors and suggests that goldfish possess two genes encoding the IR. This was not unanticipated. Two distinct cDNAs encoding the goldfish IGFR-1 receptor were isolated in parallel with the IR cDNAs, and multiple cDNAs encoding the IRs have been amplified from salmon tissues using the PCR primers described here.9 Multiple genes in fish are believed to reflect an ancient genome duplication event, perhaps as recently as 16 million years ago.21 The relatively low degree of identity between genes encoding the same protein is interpreted to show that once duplicated, each gene accumulates mutations independently.

Insulin (as well as IGFR-1) is mitogenic for neuronal progenitors in the retinas of fish.1,22 Boucher and Hitchcock1 showed that both insulin and IGFR-1 stimulate proliferation of cells in the

### Table 1. Comparisons of Amino Acid Identity among gFIR-1 and -2 and Insulin-Receptor Genes

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<th></th>
<th>gFIR-1</th>
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Receptors are from salmon (SIR), turbot, and human and IGFR-1 and -2 from goldfish.

**Figure 2.** Northern blot analysis showing tissue expression of gFIR-1 and -2 (A and B) Expression patterns of gFIR-1 and gFIR-2, respectively. The apparent band at 4 kb is background hybridization to the 28S rDNA.
CGZ; each peptide was potent at concentrations as low as 1 nM, and both produced a response similar in magnitude. Based on these observations, it was suggested that each peptide acts through its cognate receptor. The results from the in situ hybridizations (see Fig. 3B) confirm this suggestion. Retinal progenitors within the CGZ contain mRNA encoding the IR and presumably express functional receptors. Insulin is a mitogen for retinal progenitors in embryonic birds, and insulin receptors bind IGF-1, but not insulin, with high affinity (although see Ref. 30) and may regulate a tissue’s sensitivity to insulin by sequestering some IRs in an inactive form.26 Hybrid receptors, if present, represent another level of complexity of insulin-regulated events in the teleost retina.

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References


