Expression of the Insulin Receptor in the Retina of the Goldfish

Peter F. Hitchcock,1 Deborah C. Otteson,2 and Paul F. Cirenza1

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.1 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 differentiating 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 insulin and/or neuronal progenitors in the retina of the goldfish may contain hybrid IGF-1/insulin receptors. (Invest Ophthalmol 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 expansion2 and the addition of new nervous system. The retina of the goldfish grows continually by both a balloon-like expansion2 and the addition of new nervous system. The retina of the goldfish grows continually by both a balloon-like expansion2 and the addition of new nervous system. The retina of the goldfish grows continually by both a balloon-like expansion2 and the addition of new nervous system. The retina of the goldfish grows continually by both a balloon-like expansion2 and the addition of new nervous system.

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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 (rediPrime II; Amersham Pharmacia Biotech) and washed the next day in 2× SSC/0.5% SDS at room temperature for 30 minutes, followed by a wash in 0.1× SSC/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× SSC/0.5% SDS and reprobed. Uniformity of loading was verified by ethidium staining of gels and/or staining membranes after transfer with methylene blue.

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). Plasmids containing gfIR-1 and gfIR-2 (see below) were linearized, and digoxigenin-labeled riboprobes (both sense and antisense) were synthesized by in vitro translation using a DIG-RNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany). Without further processing of the riboprobes, in situ hybridizations were performed using methods published previously. The signature sequence identifying the intracellular kinase domain (GxGxxGxK) 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 primers amplified cDNAs with homology to vertebrate tyrosine (T) and cysteine (C) residues, the proteolytic cleavage site that separate the α- and β-subunits of the insulin receptor (RRR), and the signature sequence identifying the intracellular kinase domain (GxGxxGxK). 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.

**Figure 1.** Alignment of deduced amino acid sequences of IRs from goldfish, human, salmon, and turbot. Highlighted in bold are conserved tyrosine (T) and cysteine (C) residues, the proteolytic cleavage site that separate the α- and β-subunits of the insulin receptor (RRR), and the signature sequence identifying the intracellular kinase domain (GxGxxGxK). 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.
sequences for both the IGF-1 and the IRs. (A description of the goldfish IGF-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 \( \alpha \)-domain, the transmembrane domain, and a portion of the intracellular \( \beta \)-domain. In addition, both clones contain sequences common to all receptor tyrosine kinases, including conserved cysteine (C) and tyrosine (Y) residues, the tetrabasic proteolytic cleavage site (RRRR/RQRR), and the tyrosine kinase signature sequence \( (GxGxxG21xK).12 \) and cDNAs for the two goldfish IGF-1 receptors showed that the goldfish IR was more similar to IR homologues from other animals than to goldfish IGF-1R (Table 1), consistent with the interpretation that \( gfIR-1 \) and \( gfIR-2 \) are distinct from the cDNAs encoding the IGF-1 receptor. Further, comparisons between \( gfIR-1 \) and \( gfIR-2 \) showed that they are only 69.7% identical (Fig. 1 and Table 1). We interpret this difference in sequence identity, at both the nucleotide and amino acid levels, to indicate that \( gfIR-1 \) and \( gfIR-2 \) 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 \( gfIR-1 \) hybridize 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 kinases\(^ {12} \) and is most similar to the receptor for IGF-1.\(^ {17} \) Although principally studied in mammalian systems, IRs are expressed in various fish tissues,\(^ {18} \) including the brain\(^ {19} \) and retina (see Results). Unlike mammals (and birds), however, insulin is not synthesized in the teleost brain,\(^ {20} \) 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 IGF-1 receptor were isolated in parallel with the IR cDNAs,\(^ {11} \) 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 IGF-1) is mitogenic for neuronal progenitors in the retinas of fish.\(^ {1,22} \) Boucher and Hitchcock\(^ {1} \) showed that both insulin and IGF-1 stimulate proliferation of cells in the

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**Table 1. Comparisons of Amino Acid Identity among \( gfIR-1 \) and \( -2 \) and Insulin-Receptor Genes**

<table>
<thead>
<tr>
<th></th>
<th>( gfIR-1 )</th>
<th>( gfIR-2 )</th>
<th>SIR-1</th>
<th>Turbot IR</th>
<th>Human IR</th>
<th>( gfIGF1R-1 )</th>
<th>( gfIGF-G2 )</th>
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<tr>
<td>( gfIR-1 )</td>
<td>100</td>
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<td>60.7</td>
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<tr>
<td>( gfIR-2 )</td>
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<td>65.4</td>
<td>55.5</td>
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<td>68.9</td>
<td>47.6</td>
<td>50.7</td>
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<tr>
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<td>-</td>
<td>100</td>
<td>76.1</td>
<td>61.2</td>
<td>44.9</td>
<td>48.4</td>
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<tr>
<td>Turbot IR</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>60.5</td>
<td>47.0</td>
<td>49.5</td>
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<tr>
<td>Human IR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>46.9</td>
<td>49.5</td>
<td></td>
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<tr>
<td>( gfIGF-1 )</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>100</td>
<td>68.2</td>
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</tr>
</tbody>
</table>

Receptors are from salmon (SIR), turbot, and human and IGF-1 and -2 from goldfish.
regulating the persistent mitotic activity in the retinas of adult fish.

In addition to its mitogenic effects, insulin can have multiple other effects in the both the developing and mature nervous system.14 Insulin is best known as a regulator of glucose metabolism, and tight control of glucose utilization is perhaps the major function of this peptide in the nervous system.25 In addition, however, insulin can act as a trophic factor to promote neuronal differentiation, process outgrowth, and synapse formation.14–16,24,25 Further, insulin can modulate synaptic transmission. IRs are physically associated with postsynaptic specializations,20 and exogenous insulin alters the electrical response of neurons in the olfactory bulb22 and decreases the amplitude of the a- and b-waves of the bovine electroretinogram.24 Expression of the IR by the postmitotic cells in the retina of the goldfish suggests that among these cells insulin may have pleiotropic effects, from regulating metabolism to modulating synaptic transmission.

Finally, the cellular expression of insulin and IGF-1 receptors in the goldfish’s retina appears to be completely overlapping11; differentiated retinal cells as well as the retinal progenitors express both receptors. This suggests that a significant fraction of the insulin and IGF-1 receptors in the fish retina may exist as hybrids, dimers of insulin and IGF-1 half-receptors. Hybrid receptors are widely distributed in mammalian tissues29 and are expressed in the embryonic retina of birds.30 Hybrid receptors bind IGF-1, but not insulin, with high affinity29,31 (although see Ref. 30) and may regulate a tissue’s sensitivity to insulin by sequestering some IRs in an inactive form.32 Hybrid receptors, if present, represent another level of complexity of insulin-regulated events in the teleost retina.

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References


