Molecular cloning, identification and characterization of four distinct receptor subtypes for insulin and IGF-I in Japanese flounder, *Paralichthys olivaceus*

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Abstract

Insulin receptor (IR) and IGF-I receptor (IGF-IR) are structurally and functionally related and belong to the tyrosine kinase receptor family. In teleosti such as salmonids and turbot, occurrence of multiple IR and IGF-IR members has been reported, but the structures of a complete set of both IR and IGF-IR members in a single teleost species have not yet been characterized. In this study, we cloned and analysed four distinct cDNA clones for IR and IGF-IR members from the liver and kidney of the Japanese flounder (*Paralichthys olivaceus*). Deduced amino acid sequence analyses and phylogenetic analysis have revealed that two of them (fIR-1 and fIR-2) belong to IR members and the other two (fIGF-IR-1 and fIGF-IR-2) are IGF-IRs. fIR-1 and fIR-2 comprised 1369 and 1368 amino acid residues respectively, and fIGF-IR-1 and fIGF-IR-2 comprised 1412 and 1418 residues respectively. All the receptor proteins contained

cysteine-rich domains in their α -subunits, and conserved each transmembrane and tyrosine kinase domains in their β -subunits. The amino acid sequences of fIRs and fIGF-IRs showed more than 90% sequence identity with turbot IR and IGF-IR respectively. When compared with their mammalian homologues, fIGF-IR-1 and fIGF-IR-2 proteins contained large insertions at their C-termini, as was observed in the corresponding region of turbot IGF-IR. Occurrence of multiple species of mRNA for each IR and IGF-IR was suggested by Northern blot analyses. A ribonuclease protection assay revealed diverse expressions of four receptor mRNAs in a wide range of tissues including heart, liver, ovary, testis, brain, gill arch, kidney, skeletal muscle, intestine, stomach, spleen and eye of the flounder.

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Introduction

Insulin receptor (IR) and insulin-like growth factor-I receptor (IGF-IR) are members of the tyrosine kinase receptor family (Ebina *et al.* 1985, Ullrich *et al.* 1985, 1986, Hanks *et al.* 1988). These two receptors are composed of two α - and two β -subunits linked by disulphide bonds, forming $\alpha_2\beta_2$ heterodimers. The α -subunits compose their extracellular domains and possess the cysteine-rich ligand-binding sites. The β -subunits contain single transmembrane domains and highly conserved cytoplasmic tyrosine kinase domains (Cheatham & Kahn 1995, LeRoith *et al.* 1995). In teleosts, ligand-binding studies using mammalian IGF-I and insulin have consistently demonstrated the presence of high-affinity binding sites on various membranes prepared from teleost tissues (Drakenberg *et al.* 1993, Párrizas *et al.* 1995, Maestro *et al.* 1997). The binding of IGF-I in teleost muscle ectotherms was abundant and at higher affinity than that of insulin, although IGF-IR mRNA was expressed at the lowest levels in rainbow trout muscle (Greene & Chen 1999a,b).

However, in birds and mammals, the situation is reversed, suggesting functional changes in insulin and IGF-I during evolution (Gutiérrez *et al.* 1995).

Insulin and IGF-I show overlapping effects in the growth promotion of teleosti (Kelley *et al.* 1993, Cheng & Chen 1995), but the mechanisms of their receptor-mediated signal transductions are largely unknown. In tetraploid salmonids, multiple IR and IGF-IR species have been identified by cDNA cloning. Chan *et al.* (1997) have suggested the presence of four IR and two IGF-IR mRNA species in the salmon by cloning partial cDNAs for their regions of the tyrosine kinase domain. In rainbow trout, three IR and two IGF-IR cDNA clones have been isolated (Greene & Chen 1999a,b), although these clones do not encode full lengths of each protein. Recently, one cDNA clone encoding a partial amino acid sequence of turbot IR and another clone encoding the full sequence of turbot IGF-IR have been reported (Eliès *et al.* 1999). For understanding the distinct or common physiological functions of insulin and IGF-I in teleosts, it is essential to elucidate the complete structures of all IR and IGF-IR

Table 1 Primer sequences used in the cDNA cloning

Primer no.	Sequence	Nucleotide position
1 (sense)	5'-GACCTCTTCCCCAACCTCACGGTCATCCGCGG-3'	337-368 hIGF-IR
2 (antisense)	5'-CATCGGCTTCTCCTCCATGGTCCCTGGACA-3'	589-618 hIGF-IR
3 (sense)	5'-GAGTTTCTCAACGAAGCTTCTGTGATGAA-3'	3181-3209 hIGF-IR
4 (antisense)	5'-CATCTTGGGGTTATACTGCCAGCACAT-3'	3784-3810 hIGF-IR
5 (antisense)	5'-CACCGAGTCTAGGATCTTGGACCACTCCA-3'	1446-1476 flR-1
6 (sense)	5'-TGGTCGTTTGGGGTTGTGCTGTGGGAGATC-3'	4747-4776 flR-2
7 (sense)	5'-ACCCTGAGCTCTGCTACTTGGCCACCTTGG-3'	1419-1448 flR-1
8 (antisense)	5'-CCCAATGTGTGTGGAGGAAGTGTGCACT-3'	5301-5330 flR-1
9 (sense)	5'-TCCATTTGAACTGTCTCTCGCTAAAGG-3'	1071-1100 flR-2
10 (antisense)	5'-GGTCTCTGTGGACGTGCTTCTTGGCGTTGA-3'	4389-4418 flR-1
11 (sense)	5'-AGCACCAGGTCCACGGAGCCAAGAGCGAGA-3'	2945-2974 flR-2
12 (antisense)	5'-TAGGACGACGGGTCCAGCGGGATGCTCTCC-3'	5061-5090 flR-2
13 (antisense)	5'-GTTGTACAGGCCAATGTCTTCAGGCTGGT-3'	1008-1037 flGF-IR-1
14 (sense)	5'-CTCATTATGGATGCGGAGTTCAACAACATT-3'	1116-1145 flGF-IR-1
15 (sense)	5'-ACCACCCAGGTCTACCCCCACTCAAAAAA-3'	3918-3947 flGF-IR-1
16 (sense)	5'-GGGGACAAAAACAATAATATCAACCTG-3'	982-1011 flGF-IR-2
17 (antisense)	5'-ACATCCTCATCAGTCAAAACAGCATGTC-3'	4329-4356 flGF-IR-1
18 (sense)	5'-GGGGTTGTACTGTGGGAGATTCCACTCTG-3'	4396-4425 flGF-IR-2
AP-1	5'-CCATCCTAATACGACTCACTATAGGGC-3'	Adaptor primer 1*

hIGF-IR, human IGF-IR; *Marathon cDNA Adaptor primer 1.

species in a single teleost species. In the present study, we have cloned and characterized the complete set of cDNA for IR and IGF-IR (two IRs and two IGF-IRs) and analysed their tissue distribution in a diploid fish, Japanese flounder (Tabata 1991).

Materials and Methods

Animals

Sexually matured male and female Japanese flounders (*Paralichthys olivaceus*), weighing 700–900 g, 15 months of age, were purchased from a local fish farm (Yada Suisan, Suzuka, Japan). At the time of the experiment, it was observed that the gonads were fully developed in both sexes. The liver, spleen, stomach, intestine, testis, ovary, kidney, skeletal muscle, brain, heart, gill arch and eye were quickly removed from the fish and immediately frozen in liquid nitrogen and kept at –80 °C until use.

RT-PCR and cDNA cloning

Total RNA was extracted from tissues of Japanese flounders by the acid guanidinium–phenol–chloroform method (Chomczynski & Sacchi 1987) and poly(A)+ RNA was isolated with Oligotex-dT30 (Takara, Tokyo, Japan). cDNA libraries were prepared from liver and kidney poly(A)+ RNAs with a Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA), and IR and IGF-IR cDNAs were amplified by PCR with sense and antisense primers (Table 1) derived from the human IGF-IR cDNA (Ullrich *et al.* 1986). The 5'- and 3'-regions of IR and

IGF-IR cDNAs were synthesized by the rapid amplification of cDNA ends method. The amplified cDNA fragments were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequenced by the dideoxy chain-termination method (Sanger *et al.* 1977).

Ribonuclease (RNase) protection assay

Appropriate cDNA fragments for flounder IR-1 (flR-1), flR-2, flGF-IR-1 and flGF-IR-2 were synthesized by PCR and cloned into pGEM-T EASY vector. The antisense RNA probes were synthesized by using SP6 and T7 RNA polymerases and [α -³²P]CTP. The RNA probes (5×10^4 c.p.m./assay) were hybridized with 20 μ g total RNA at 50 °C for 16 h in 30 μ l of the solution containing 40 mM Pipes (pH 6.5), 0.4 M NaCl, 1 mM EDTA and 80% formamide. The samples were digested with RNase A (40 μ g/ml) and RNase T1 (2 μ g/ml) at 37 °C for 30 min and then incubated with proteinase K (50 μ g/ml) at 37 °C for 15 min. The RNA fragments protected from RNase digestion were separated by electrophoresis on a 6% polyacrylamide–7 M urea gel and detected by autoradiography. The radioactivity of the protected fragments was analysed with a BAS1000 imaging analyser (Fuji Photo Co., Tokyo, Japan).

Northern blot analysis

Poly(A)+ RNA (4.5 μ g) isolated from flounder heart was separated by electrophoresis on a 1.0% agarose gel containing 2.2 M formaldehyde and transferred to GenScreen membranes (New England Nuclear, Boston, MD, USA).

Figure 1 Schematic representation of the cDNA structures of the flounder Irs (fIR-1 and fIR-2) and IGF-Irs (fIGF-IR-1 and fIGF-IR-2). The boxes indicate the coding regions, and double lines mark the 5'- and 3'-non-coding regions. The signal sequence is shown by a dark band at the 5'-end of the coding region, the cysteine-rich domain by cross-hatching, the transmembrane domain by a vertical thick bar, and the tyrosine kinase domain by a black box. The thin horizontal lines indicate the cDNA clones isolated. The closed arrowheads show the position of the primers used for PCR. The thick horizontal lines show the probes used for Northern blot analysis. The broken lines show the regions used for the RNase protection assay. The nucleotide sequence of each cDNA will appear in EMBL, GenBank and DDBJ with Accession numbers; fIR-1: AB065096, fIR-2: AB065097, fIGF-IR-1: AB065098, fIGF-IR-2: AB065099.

(a)

fir-1	1	---MRRWTLKLRACIVFTTICTCLICCOGLINGEICPSKDIRNNVTTLQSLNCTIIEGHLKILLMFKTKAEDFRGLSYPKLRVVDYVLLFRVYGLEMLS	100
fir-2	1	MVS.TEPVMCPRITLC.VLTAV.PMVS.HRAT...V.....NM.T.....V.....R.P..H..F.....L.....SFR	100
Turbot		-----	0
Human	1	MGTGGRRGAAA.PLLVAVAAALLGAAGH.YP..V..GM....L.R.HE...SV...Q.....RP...D.F...IMI..L.....S.K	100
fir-1		ELFPNLTIVIRGNLFFNYALVIYEMLQLKEVGFFYSLMNITRGAVRIEKNPELVLATLDWSKILDSVEDNYIVANKNERE---GDCVCPGTAQGEIIPQNT	197
fir-2		D.....VF.....R.T.LH.....D.....S.....I.....D.....A.L.KT.TOTT.	199
Turbot		-----L.LH.....D.....T.....F.....D.....Q.T.S.	72
Human		D.....SR.....F..VH...L.L.N.....S.....N.....I..R.....L..DDN.E...I.....K.KTN..ATV	200
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fir-1		INGHFRRRWSONHCQRMCLDNKHGGTTLQGGCCHDQCLGGCSVPSNASSFVACMLNQHGNSTVERPPGGYVFRGWRGVSFSTQELHNQKQTKKLN	297
fir-2		S.E...T.K..K..PVO...RA..KDD.....LQ.NS..H...RG..LEGN..D..ENHFT.K...T.A...D...K..RE.ERA	299
Turbot		G...VAD...A.S.....E.G.....R.....K.A.....N.SLV.NL	172
Human		Q.VE...THS...KV.PTI..SH...AE.L...SE...N..Q.DDPTK...R.FYLDGR...T...P..H.QD...N...D..HK..NS---	296
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fir-1		QDRELSCNEYVIHNGAIPPCPSGYTTINSTLLACSPCAGLSPKLVGN---KTIDSVNSAQLRGCTVLHGNMIKIRGGNNIAAEASLGQLEEITG	394
fir-2		KNPD---H.....VAD...V..SS.N.M...V.M.L---V..TA...K...N.SLV.NL	394
Turbot		SG.....G.....TL.....V..T.....I.....	269
Human		---RQC.HQ....NK.....MN--SN..L.T..L.P..V.HLLEGE...T...E...IN.SL..N.....L.....N..LI...S.	393
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fir-1		YLTVRRAVALVSLFLRKLRIIRGEHLETDVHAFYALDNQNLRELWDWSKHNLTLRGRMFFHYSNKLGMSEIRKMEEVGTGKDRNQKNGIAVRNNGDQA	494
fir-2		S.....F...V...EQ.IGNYS.....Q.....K...Q...L.....RW.....Q..MD..TKT.	494
Turbot		IY.....Q...V.....D...X..D.....	369
Human		..KI..S.....F...L...T..IGNYS.....Q.....TQ.KL...P..L..H...S...G.QER.D..LKT....	493
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fir-1		SOETKLLKFTVIKTTSSNMIMLKWEPFWPLDFRDLGLFMVLYKEAPYKNVTEFDGQDAGCSNSWAIADVDPPPR---DGKKAEDPRHLIRLPKPWTQYAIM	591
fir-2		..NHPP...Q.R.M.DK..V..A...S.Y.....FR.....V.....R.APDGE.NQLE.G...L.....	594
Turbot		..M.....V.T.....S.....S.ST---G...T.R.....	468
Human		..NE...SY.R..FDK.L.R...Y..P.....LF.....Q.....TVV..I...L.SN--P..SQNH.GW.M.G.....F	592
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fir-1		VKTQLSASDEHQVHAKSEIIVVRTNASKSPVLPDISSSNSSSQIILKWKPPKTPSPNGNITHYRVICRKQPEDSDLYKFDYVLQGMELPSRTP-PTWTVR	690
fir-2		..S.....STQ...A.....ND.....L.F.QR...A.E.....QK..K...V..TQVDS	693
Turbot		X.....K.....A.....THLDS	567
Human		..LVTF...RRTY...D...Q.D.TN...V.....SD...L.FWER..A.E.FEL...K.LK...WS.FFESE	692
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fir-1		MSRSGTTQINPPRG-KVCAQPKTDNQLKKEQEEIEYRKTFENYLHNEVFESRPTRRRSVGVANATLLPNFLTAAAPSFPFGSTTASPDDDDKDDEGSKVE	789
fir-2		EEHKWNQTEEGS.TR...EKE...K.DS.....IKRS.Q...AAIDI.NRTYFSH.T...L.E.THIPDE.T---F.ST.TV	789
Turbot		DEQKWNHTDE.TS.G.C...S.S...S...Q...TV..LS...T..E---	665
Human		D.QKHNQSEYEDSAGECS...S..IL..L..SSF...D...V..VP..S.K...--SLGDVGNTVAVPTVAA..NT.S.SV.TSP---.EHRPF	786
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fir-1		LKVSFESKESKVISNLHHFTSYKIEIMACNHVTDIKRSMTATVVSARTMPEEKADDIPGHVTHEIVTAEPYVLIKWNAPRSPNGLIILYEVFIRKVGDTVE	889
fir-2		VT.HA...T....R...Q...H...PK.PE...S.....D...V.PISS.VTENS---H.T.LE.AA...M.....N.KRL...E	886
Turbot		L...T.....P..L.....E..P.....A.....T.....	765
Human		E..VN...L...G.R...G.R..LQ...QD.PEE...V.A.....A...V.P...FENN--V.HLM.QE..KE...V...S..RY...E	883
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fir-1		HTTVSRPAYLKTGGTKLSLVQPGNYSVRVRATSLAGNSFTETTYFFMPNPDP---GLNIVIGVPVFCIFILLFVGGAFFWIKKRTQEGTGPLIASS	986
fir-2		LHY...NMFKVNR.C..KVMH...T..I.....W.P..YVQDASDP-LYIVK...I.....TVIG.VFF..N..Q..S..IY..	985
Turbot		..A.....A.....A.....I.....F..K.....	862
Human		LHL...KHFALE..CR.RGLS...I.....W.P..YVTDYLDVPSNIAK..I...LIFVF.FSV..I..SIYLFIR...PD..L..Y..	983
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fir-1		NPEYISAN-----DVYVPDEWEVPREKITVIRELGQGSFGMVYEGIAKDIVKGPDPTRVAVKTVNESASLREIEFLNEASVMKAFSCHHVRLLGVS	1081
fir-2		L...T...EE...A...AI...I...T...N...I..ESE.....	1080
Turbot		I.....	957
Human		L..SDVFPCS...S...L...N.R..I..EAE.....G..T.....	1083
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fir-1		AQPTLVVMEMLTHGDLSYLRNLRPDSENNPTGRPPPTLKEMIQAHAELADGHAYLNAKKHVHRDLAARNQVVAEDYTVKIGDFGMTRDTYETDYRKGG	1181
fir-2		G.....F..S..A.....F.....N..L.....	1179
Turbot		C..S.....F.....F.....	1057
Human		G.....A.....S...EA...-.....Q.....F.....H.F.....	1182
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fir-1		KGLLPVRWMAPELSKDGVTFAHSDCWSFGVVLWEISTLAEQPYQGLSNEQVLKVFMDGGYLDLPENSPERMHNLMMQWQYNPKMRPMHEIIEMLREDL	1281
fir-2		S.....D..AD..I.....A.Q...Q.....	1279
Turbot		P.....S.....Y.....	1157
Human		TS..M.....TS.....Q.D...VTD..R...F..N...T..L..VNL.KD..	1282
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fir-1		HPSFQEVSFYFSEENKVPETEFDLDDLNMESIPLDPSYSQREBSLGRSGPSVALRG-NYEEHVPYTHMNGGKNRILSLPRSSPS	1369
fir-2		L.....P.VS.D...ME.....QC.D..E.S.MG...SYE.H.I.F.....T...G.....	1368
Turbot		X.....LGG.....	1245
Human		P.....H...A..S..LEMEFED..NV...R..HC...AG...G.S.LGFKR-S...I.....T...N...	1370
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figF-IR-1	1MRSGSLMGSTTLFWSLMLSVSTICIWPTYGEITGGPIDIRNDISEFKRLN	TVVEGYLQILLINDKTN-NIHQEVFRSLSPKLTITIDYLLFRVSGL	99				
figF-IR-2	1..RTERNRL...G...GL...SLLR.ATA	...S...G...R...S...	99				
Turbot	1..RAEWNR...G...GL...S.HR.AAAK	...S...G...T...R...	99				
Human	1.K...GG...P.SL.G.LFLSAALS...S...	...YQQL...I...H...SKAED-----Y.YR...V.E...A...	93				
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figF-IR-1	DSLSMLFPNLSTIIRGRQLFYNYALVIYEMTS	LKDIGLYNLKNITRGATRIEKNPEL	YLDSDVDSLMDAEFNININGNKKAKE	DNVPGIMEDNPIK	199		
figF-IR-2	...A...AV...N...F...	...R...I...	...I...Y.A...QS...	SD...NS.Q...	199		
Turbot	...T...TV...S...F...	...R...I...	...I...Y.A...QS...	SD...NS.Q...	199		
Human	E.GD...TV...WK...F...N...	...R...I...AD...	...ST...L.VS...Y.V...PP...	GDL...T.EK.MAE	193		
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figF-IR-1	RTLFDNDYDRTWSTOCQKV	PEHCK-FASTDKGESH	SOGLGTSTEPNNDMA	SASLHYFHEDR	VDPPLGTYPKESWR	ITMDLSQVHLPGD---	295
figF-IR-2	K.M...N...N.NH...	E...K.VRR...	AD...P...S.A.YS.T.A...	M...Y.QG...	A...P...G...SAE...	K...EF---	296
Turbot	K.M...S...N...N.NK...	E...R.ARQ...	AD...P...S.A.GS.T.A...	V...Y.QG...	A...P...G...SAE...	K...EF---	296
Human	K.TI.NE.N...TNR...	M...ST.GKR...	ENN...PE...S.SA.D.T.V...	R...YAGV...	A...PN...R...G...VDR.F	ANILSAESSDS	293
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figF-IR-1	POFVIHGGEMHDP	PSGFTTRNETNRMF	SACNGLDKVCTPP	---IIDSVDAAQSLKD	TVIRGNLDINIRRGNNIASELES	FMGLIQTQVKGVIKHS	391
figF-IR-2	DS.I...S...TTP.S.N.T.	D...EEK---	V...M...G...	K...H...H.MVA...	TC...R.T...GS...		392
Turbot	DS.I...E...PE...Y...TTPDS.T.T.	D...EEK---	V...M...G...	K...H...H.MVA...	T...KR.T...R...		392
Human	EG...D...QE...I.GSQS.Y	IP.E.P.P...	BEEKTKT	'...TS.M.QG...	IFK...L...N...	EV.T...	393
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figF-IR-1	HALGSLSFLKSLHYINGQELIDNMYSFSA	INNOHLQYLWDWSQHNLTRAGRLFRNPKL	EMPEIHTMWEKTKITVKPEEG	---DFRNGDRAS	ESHTL		489
figF-IR-2	.T.S...AS.R...R.TD.ED.L.D.AS...	PD.Q...K.R...KT.K.S.A...	RS...RN.D.G.QGRFD...	Y...	TI...		490
Turbot	.T.S...A.RG.R...D.EV.L.D.A.A.FD...	Q...K...A...TS...RN.D.G.RGRFD...	S---	TI...			490
Human	...V...N.R.L.L.E.QLEGN...	YVLD.N.Q...DHR...K.KMY.AF...	VS...YR.E.V.GTKGRQSK.DINT...	E...	DV...		493
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figF-IR-1	PFKTNETTSHITRLTWDRYQPPDFGDLIS	FIVYFKESPFQNTITEPDGQD	GSNSWHMVDVLPQDKTSEPNVSLPHLKP	WTQYAI	SVKAITLQVED-KH		588
figF-IR-2	N.S.T.S...TR.K...Q.R...YR...	Y...Y...E...	N...E.RL.D.D.G.L.SA...	F...M.K...			589
Turbot	K.SSS.S...TR.K...Q.R...YR...	Y...Y...E...	I...E.RP.DLD.G.L.SS...	VF...M...			589
Human	H.TSTT.SKNR.II.L.R...YR...	T.Y.A.K.V.Y...A...	N...PN.DV...GIL.HG...	VY...V...TMVEND.			593
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figF-IR-1	ITGAKSDIYIRTRPSSPSVPKDARAFANS	STKLIVKWSPPVFNGLNTYILVRWQQO	PEDRELYOHNY	SKELKIPIRISATGLTMDMDNTKPHQVRLG			688
figF-IR-2	MPT...KVV...S.V.M.Q.V.YS...	T.Q...R...TA...	Q...A...	A.I.VG.QEED...	TKPDPE		689
Turbot	MPS...KVV...S.A.M.Q.V.YS...	Q...R...S...Q...	A...	A.I.VG.QEED...	TKPDPE		689
Human	.R...E.L...NA.V...L.L.VLSAS...	SQ.L...N.SL...S...T...R.Q.GY...	R...DK...	KY.D.TI.IEEV.ENPKTEVC			692
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figF-IR-1	GGREGPMVAED-AEEKDREKDDRVFLKIFEN	FLHNAIVLPRPPRRRR	DVGVANDTLFHSAGKGTTLGPGNSTD	GVP-----P--	IKEYPFME		777
figF-IR-2	..DK..C.PCPKSV.DLEA.AA.ASYR.V...	KS.FT...	L.I.KS.HSRRRLH.T.SSNV.PHQAA.N--	SSNLADVEPADR.FE...			787
Turbot	..ADK..C.PCPKSV.DLEA.AA.ASYR.V...	S.FT...	L.F.S.HSRRRLH.T.SSHV.PHQAA.NNSSTTAE	EQADR.FE...			789
Human	..EK..C.PACPTEA..QA..EEAEYR.V...	S.FV...E.K...	MQ...T.MSSR.RMTTAAADTYNITDPELET	-----F.			779
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figF-IR-1	DKSSAEYLDIPN-LQPFVYRLDIHA	NEEVGR--SAGAFVFSRTKPAVKADDIPG	KVIYERSDKVEGSVVLHWPEPIMP	NGLILMYEIKFRIGTEPEK			874
figF-IR-2	QAVTERE.L.SG...	...RQ.Q...AE...	...Q.TV.G---H.DW.F.R...G.PH...	K.AS.T...			881
Turbot	QAV.ERVQIFD--...I...RQ.Q...	AE...	...Q.TW.G---H.DW.F.R...PH...R.V...	K.AA.T...			882
Human	SRVDNKERTVISN.R...L.I...S...H.AEKLGE	SN...A.M.EG...	P.TW.P--RP.N.IF.K...EN...	YGSQV--DQ			876
	* * * * *						
figF-IR-1	HEVSRQHREHRCARLTNLSSGNSYARVRAT	SLAGNSWTESVFFVYPPPKRDDGVAFY	VIIPILATLLIASLTITLIFVNV	KRNSDRIGNGVLIAS			974
figF-IR-2	...G.M.QAQ.V...S...P...V...	HI.DL.AERYEN--VL	AM.F...ATIF.CL.V.M.VAF...	L			978
Turbot	...G.T.QNQ.V...S...P.H.V...	NA.DL.AERYEN--VL	AM.F...ATIF.CL.V.M.VVL...				979
Human	R...E...KYG...K.NR.NP...T.IQ...S...	DP...QAKTGYEN-FIH	I.AL.VAVI.IVGG.VIM.YVFHR...	NS...			975
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figF-IR-1	AAAAVNPEYFSAAEYVPDWEVARGK	ITMKELGQGSFGHMYEGLAKGVKDEP	TRVAIKTVNESASMRERIEFLNEASVMKEFN	SHVVRLLGVVSQGOPT			1074
figF-IR-2	...D...D...V.E.VLSR						1078
Turbot	...D...D...E.ALSR						1079
Human	...DV...E...E...						1075
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figF-IR-1	LIVIMELMTRGDLKSLHRSR--KENST	QVLPPLKKMIQMAEITADGMAYLNANKFVHRDLAARN	MAEDFAVKIGDFGMDRIDYETDYRKGGKGLP				1172
figF-IR-2	...Y...Y...PKQOWSSLS...	L...L...Q...	D...T...				1178
Turbot	...Y...Y...PKQOWSSLS...	R...L...Q...	D...T...				1179
Human	...Y...Y...PEM...NPVLAP.S.S...						1175
	* * * * *						
figF-IR-1	VRWMSPESLKDGVTMTSDVWSFGVVLWE	ITLAEQPYQGSNEQVLRFVMEGGLDKPDN	PDMLFELMRM	WQYNPKMRPSLEIISIKDELDSFPFG			1272
figF-IR-2	...N...N...S...L...L...E...Q...						1278
Turbot	...H...H...S...L...L...V...E...Q...						1279
Human	...Y...Y...L...L...L...E...MFG.R						1275
	* * * * *						
figF-IR-1	EMSPFYSEENKPPDTEELDMEVEN-MENI	PLDPASTRQPSAVAAPSSGCTGGTPPPSAQQLSPM	QGPSTPLLPVSPSSSGPVASALASPGQALDKHSGH				1371
figF-IR-2	.V...AD...SEEQQVHLDKMDNI.DV.A.QP	ST...QQAQV.QQTPSPSSSEAPPAPSLSPSS	.S.CTSTAAMDQASGQ-QA.NGLSGPLAA.S				1377
Turbot	DS...AD...V.DPQVHQDKMDSVDDV...	P.ST...QQSPV.QQTPPPSSSEAPPAPSLSPSS	.S.CTSTAAMDQASGQ--SGNGLSGPSHAA.S				1377
Human	.V...Y...L.EP...L.P...SV...SASS--	SLPL.DRHSG--	KAE				1326
	* * * * *						
figF-IR-1	VSANGPVVVLRPNFDEMOPYAHMNGGRK	NERLALPQSSA					1412
figF-IR-2	GLGT.AGGTM...SLE.LP...	M...					1418
Turbot	GLGTSAG.AMC.SLE.LP...	M...L...					1418
Human	NGPGPG.L...AS...R...	T...					1367
	* * * * *						

The membranes were prehybridized with yeast RNA (200 µg/ml) at 42 °C for 2 h and then hybridized with radiolabelled DNA fragments derived from 5′- or 3′-non-coding regions of each IR and IGF-IR cDNAs (Fig. 1) in 50% formamide, 6 × SSPE and 0.1% SDS at 42 °C for 16 h. After washing twice with 2 × SSC containing 0.1% SDS at 60 °C for 30 min, the membranes were subjected to autoradiography for 14 days at −80 °C.

Results

cDNA cloning and characterization of the deduced amino acid sequences for fIRs and fIGF-IRs

Two fIR cDNA clones, fIR-1 and fIR-2, were isolated from the liver cDNA library, and two fIGF-IR cDNA clones, fIGF-IR-1 and fIGF-IR-2, were obtained from the kidney cDNA library. Figure 1 shows schematic representations of the structures of these four cDNA clones. The fIR-1, fIR-2, fIGF-IR-1 and fIGF-IR-2 cDNAs contained open reading frames (ORFs) encoding proteins comprising 1369, 1368, 1412 and 1418 amino acid residues respectively. The deduced amino acid sequences are shown in Fig. 2, compared with those of human and turbot IRs and IGF-IRs. A potential proteolytic cleaving sequence, R-X-R-R, was conserved at the putative junctions of α- and β-subunits of all these receptors. The cysteine-rich domains in the α-subunits of both fIRs contained 26 cysteine residues whereas both fIGF-IRs contained 24 cysteine residues, as was observed in α-subunits of mammalian IRs and IGF-IRs respectively. In the β-subunits of fIRs and fIGF-IRs, a putative IRS-I-binding site, NPEY, a potential ATP-binding site, G-X-G-X-X-G-21X-K (Hanks *et al.* 1988) and an autophosphorylation site, YETDYY were well conserved in their tyrosine kinase domains. As compared with human IGF-I-IR, large insertions were observed in the C-termini of fIGF-IRs and turbot IGF-IR (Ullrich *et al.* 1986, Eliès *et al.* 1999).

As shown in Table 2, both fIRs showed higher sequence identities with human IR than with human IGF-IR, and both fIGF-IRs exhibited higher sequence identities with human IGF-IR than with human IR. Turbot IR showed a higher sequence identity with fIR-1 (91.7%) than with fIR-2 (73.2%), and turbot IGF-IR showed a higher sequence identity with fIGF-IR-2 (90.5%) than with fIGF-IR-1 (66.8%), suggesting that

Table 2 Amino acid sequence similarity between human, turbot and flounder IRs and IGF-IRs

	Receptor	fIR-1	fIR-2	fIGF-IR-1	fIGF-IR-2
Human	IR	65.6	67.5	52.3	51.8
	IGF-IR	55.4	56.9	63.6	63.0
Turbot	IR*	91.7	74.0	50.6	50.7
	IGF-IR	51.1	51.0	66.8	90.5
Flounder	fIR-1	100	73.2	52.1	51.3
	fIR-2	73.2	100	51.0	51.4
	fIGF-IR-1	52.1	51.0	100	67.5
	fIGF-IR-2	51.3	51.4	67.5	100

*Because a lack is seen in turbot IR N-terminal end, fIR-1, fIR-2, fIGF-IR-1 and fIGF-IR-2 comparisons with turbot IR were performed using 1369/1245, 1368/1245, 1412/1245 and 1418/1245 amino acids respectively.

fIR-1 and fIGF-IR-2 are the flounder counterparts for the reported turbot IR and IGF-IR respectively. In phylogenetic analysis, the evolutionary positions of fIR-1 and fIR-2 were located in the IR groups, whereas fIGF-IR-1 and fIGF-IR-2 belonged to the IGF-IR groups (Fig. 3).

Northern blot analyses of transcripts for fIRs and fIGF-IRs in flounder heart

Figure 4 shows the results of Northern blot analyses of mRNAs for fIRs and fIGF-IRs in the heart, where abundant expression of all the four mRNAs was observed by the RNase protection assay. One major transcript band of 13 kb was detected for fIR-1, and one major mRNA band of 8 kb was observed for fIR-2. Two major bands of 11 and 9 kb were detected for fIGF-IR-1 transcripts, and one major band of 11 kb and one minor band of 0.9 kb were observed as fIGF-IR-2 transcripts.

Tissue distribution of IR and IGF-IR mRNAs in flounder

The distribution of fIR-1, fIR-2, fIGF-IR-1 and fIGF-IR-2 mRNAs in the flounder tissues were examined by the RNase protection assay (Fig. 5). fIR-1 mRNA was clearly detected in the liver, kidney, brain, spleen, testis, heart, gill arch and ovary, and faintly in the stomach, intestine, skeletal muscle and eye. The highest expression was observed in the heart. Similar results were obtained in fIR-2 mRNA analysis except for its abundant expression in the gill arch. fIGF-IR-1 mRNA was clearly detected in

Figure 2 (a) Comparison of amino acid sequences of fIR-1 and fIR-2 with turbot and human IRs. (b) Comparison of amino acid sequences of fIGF-IR-1 and fIGF-IR-2 with turbot and human IGF-IRs. Turbot and human sequences were obtained from the GenBank Database. IR; turbot (AJ224994), human (X02160), IGF-IR; turbot (AJ224993), human (M24599). Asterisks indicate identical amino acids. Gaps were introduced to maximize the matching. The signal sequences are heavily underlined, cysteine residues are shown by black boxes. Boxed regions indicate, in order, the putative precursor processing site, the transmembrane domain and the tyrosine kinase domain. The arrowheads indicate potential residues involved in IRS-1/IRS-2 binding. The open circles indicate important ATP binding and black circles indicate essential amino acids at the receptor autophosphorylation site. A black arrowhead indicates a position where insertion of exon 11 can generate different isoforms of the mammalian IR.

Figure 3 Evolutionary tree of the IR/IGF-IR family members. The tree was constructed using a distance matrix based on protein sequence and the neighbour-joining method of Saitou & Nei (1987). The amino acid sequences were aligned with the GENETYX software (Tokyo, Japan). These sequences were obtained from the GenBank Database. IR; human (X02160), rat (M29014), *Xenopus* (AJ132556), turbot (AJ224994). IGF-IR; human (M24599), rat (L29232), chicken (CAA11144), *Xenopus* (M64660), turbot (AJ224993) and *Amphioxus* insulin-like peptide receptor (S83394).

the testis, heart and gill arch, but weakly in other tissues. flGF-IR-2 mRNA was highly expressed in the testis, heart, gill arch and ovary, and in other tissues the expressions were lower. After prolonged exposure, however, all four mRNAs were clearly detected for all the tissues tested.

Discussion

In the present study, a complete set of four full-length cDNAs for distinct flR-1, flR-2, flGF-IR-1 and flGF-IR-2 were cloned and characterized. In human, a 28 amino acid open reading frame (ORF) has been identified in 5'-untranslated region (UTRs) of IGF-IR mRNA but the function has not yet been clarified. Two ORFs of 60 and 37 amino acids were observed in turbot (Eliès *et al.* 1999). In this study, ORFs of 36 and 35 amino acids were found in the 5'-UTR of flGF-IR-1 and flGF-IR-2 respectively. The short ORF was found before the polyadenylation signal which is located 250 bp upstream of the flGF-IR-2 translation initiation codon (Fig. 6). Actually, a short transcript of 0.9 kb, which may be inferred to be derived from a short ORF in the 5'-UTR of flGF-IR-2, was detected by Northern blot analysis using a probe prepared from the 5'-non-coding region of flGF-IR-2 cDNA. However, the ORF of 36 amino acid residues in the 5'-non-coding region of flGF-IR-1 cDNA had no polyadenylation signal (data not shown), resulting in the

absence of the 0.9 kb transcript expression (Fig. 6). A recent report also showed the translation of two putative unrelated proteins (leptin receptor and OB receptor gene-related protein) from alternatively spliced mRNAs which were transcribed under the control of the same promoter (Bailleul *et al.* 1997).

All the amino acid sequences deduced from the cDNA clones contained characteristic features of IR and IGF-IR. However, the varied primary structures of these four receptors suggest that similar but complex signal transductions may occur when the ligands, insulin and IGF-I, bind to them. Considering the report that the Japanese flounder is diploid (Tabata 1991) and the present findings that sequences of the 5'- and 3'-non-coding regions are less homologous than those of the coding regions between flR-1 and flR-2 or between flGF-IR-1 and flGF-IR-2 (44–48% identical nucleotide sequences as presented in DNA databases referred to in Fig. 1 legend), these four receptors may be the products of four different genes. On the other hand, molecules of insulin (Andoh & Nagasawa 1998) and IGF-I (Tanaka *et al.* 1998) have been reported to be produced by a single gene in Japanese flounder. The amino acid sequences of flR-1 and flR-2 are more homologous to that of human IR than to that of human IGF-IR, and those of flGF-IR-1 and flGF-IR-2 are more similar to that of human IGF-IR than human IR. Furthermore, the phylogenetic analysis showed that flRs and flGF-IRs were clearly localized in the IR branch and IGF-IR branch respectively in the evolutionary tree

Figure 4 Northern blot analysis of flR and flGF-IR mRNAs. Poly(A)⁺ RNA (4–5 µg) from flounder heart was electrophoresed on a 1% agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized with radiolabelled cDNA probes corresponding to the 3′-non-coding regions of flR-1 or flR-2 and to the 5′-non-coding regions of flGF-IR-1 or flGF-IR-2 cDNAs shown in Fig. 1. Positions of RNA size markers are shown at the left.

(Fig. 3). These results strongly suggest that flR-1 and flR-2 are receptors for insulin and that flGF-IR-1 and flGF-IR-2 are receptors for IGF-I.

Relatively abundant expressions of flR-1 and flR-2 mRNAs were observed at the heart, gill arch and liver. flGF-IR-1 and flGF-IR-2 mRNAs showed similar expression profiles to each other. The expression profiles of IR mRNAs are, however, distinctly different from those of IGF-IR mRNAs. Both flGF-IRs were abundantly expressed in the heart, gill arch and testis. Abundant expression was also observed in the ovary for flGF-IR-2, but not for flGF-IR-1. Varied distributions of flR-1, flR-2, flGF-IR-1 and flGF-IR-2 in diverse flounder tissues would support a wide variety of insulin and IGF-I actions. These gene duplications may have enhanced the evolution of teleosts (Amores *et al.* 1998, Pennisi 2001).

In mammals, skeletal muscle is the principal target tissue for insulin action, and the one where insulin predominantly acts to control the glucose level. IGF-I has also been reported to stimulate glucose uptake and its metabolism and protein synthesis in mammalian skeletal muscle (Dardevet *et al.* 1994). Infusion of IGF-I in humans

stimulated whole-body glucose uptake, which was similar to the response to insulin (Boulware *et al.* 1992). However, for flRs and flGF-IRs, very low levels of mRNA expression were observed in the skeletal muscle. Similar results were obtained with rainbow trout IR and IGF-IR (Greene & Chen 1999a,b) and turbot IR (Eliès *et al.* 1999). In rainbow trout, it has also been reported that glucose uptake and metabolism are higher in the heart than in skeletal muscle (West *et al.* 1993). Greene & Chen (1999b) have suggested that a potential mechanism for this discrepancy may involve IGF-I binding to cell-surface IGF-binding proteins in teleost muscle membrane.

The mechanical working of the heart requires a constant turnover of protein synthesis, as observed in rat heart. It has been demonstrated that the cardiac protein synthesis is stimulated by IGF-I and insulin in the rat (Fuller *et al.* 1992). As for fishes, both insulin and IGF-I bind more abundantly to heart than to skeletal muscle in brown trout or carp (Párrizas *et al.* 1995). The present study shows high-level expression of flR and flGF-IR mRNAs in the heart but much lower levels in the skeletal muscle. The cellular expression of IRs and IGF-IRs in flounder heart

Figure 5 Expression profiles of flR-1, flR-2, flGF-IR-1 and flGF-IR-2 mRNAs in flounder tissues analysed by the RNase protection assay. Total RNAs (20 µg) from flounder tissues were subjected to the assay with antisense RNA probes corresponding to the 3'-non-coding region in flR-1 and flGF-IR-1 mRNA, to the extracellular region of β -subunit in flR-2 mRNA and to the 5'-coding region in flGF-IR-2 shown in Fig. 1. An MspI digest of pUC19 plasmid DNA was used as a size marker. These are representative autoradiograms for each male and female from three independent experiments.

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TGCATTCATCCAGCGACGCGACCCGTTTCACGCTGCGTGTGCATCCACGGAGAGGCATAATATCAGAGGAGCTGAGCGACC 80
CCGAGCAACAAGGTCAAATCCTCCGTTGTTCCCCCCCCCTCGTTGTCGCGAAGGTGCATTACGCCACCACAACAAC 160
GCGGTGCGTGTGAGCATGATGAGCGGTGAGGAGGAGGCTGCTGCGGTCTGAAACCAGATTTGATCCTGGATATTTTCGAC 240
ACAGACGTCGTGCCGTTTCGCTTACACGGGCTGTGTTTGTGTTTGATATGATATCGTGTGCTCCTCGCCGCTCTTCCCAT 320
CCCGTTCTTGAAGGGGGGGGTGGTGTCTACCCATTCTCTGCCTCCCGAGGATCCTCCGGCAGCGGGACGTTGAGCGTTT 400
TTTCTTCCAGCTATGGAGCGTGAAGATGCGGATTACAGAGGAATCCCCGCCGCTGCTGTAGCCGATTGTACACACATTC 480
      M E R E D A D Y R G I P A A A V A A L Y T H S 23
GACACGGCGCAGTTTTTCGGCAGGAACAAGCAGAAGTATGAATACGCATTTTCAGCCTAGAGGAATAAATCGGTGATTATT 560
T R R S F R Q E Q A E L * 35
TTCTGGAGCACGGTTGGACTCCTCATTAGGAAGCGTGAGAGAGAGAGAGCTATCCCTGCTAGCCTGTGTGTAGTAACG 640
TTAGCTTTTAGCTCCTTTAGCCGGCTAACTGCTAAAAGGTGCTGGCTGGGGGATTGAAAAGCGATTTAATAATTCCGGCC 720

AGGCTTTGCCGAGCCTTTCCCCCCTCAACATATTTATTTACAGGGTCTGGGGGATTCACTTATTCCAGGAATGAGGTC 800

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Figure 6 Nucleotide sequence of the 5'-untranslated region (UTR) of flGF-IR-2 cDNA. The 5'-UTR of cDNA sequence (above) of flGF-IR-2 and the predicted amino acid sequence (below) are shown. The potential polyadenylation signal is underlined and the arrow indicates the flGF-IR-2 translation initiation codon. *Stop codon.

appears to be overlapping. The tyrosine kinase activity was stimulated by insulin and IGF-I in a dose-dependent manner in cardiac receptor preparations of carp and trout (Gutiérrez *et al.* 1995). It may be predicted that a fraction of the fIRs and fIGF-IRs in heart exist as hybrids, heterodimers of IRs and IGF-IRs, because hybrid receptors are widely distributed in mammalian tissues including the heart (Elaine *et al.* 1997). Finally, the structure of the insulin-like peptide deduced from its cDNA of the *Amphioxus*, suggests a hybrid molecule with characteristics of both peptides, insulin and IGF-I (Chan *et al.* 1990). The recent report of the existence of an insulin-like peptide receptor in the primitive chordate *Amphioxus* which shares features of both IRs and IGF-IRs clearly suggests the common origin of these two receptor species in primitive chordates (Pashmforoush *et al.* 1996).

In the flounder liver, fIR mRNAs were expressed at high levels but fIGF-IR mRNAs were at low levels. The expression pattern of IGF-IR mRNA, low in the liver and high in the gill, has also been reported in gilthead seabream (Perrot *et al.* 1999, 2000) and salmon (Chan *et al.* 1997). The role of the liver in glucose tolerance is not so clear in fish as it is in mammals. The action of IGF-I on the gill of fishes has been suggested to be for osmoregulation (McCormick *et al.* 1991, Sakamoto & Hirano 1993). fIGF-IR mRNAs were also found in the gill, suggesting an osmoregulatory action of IGF-I in that organ. Diverse distribution of IGF-IR-1 and IGF-IR-2 mRNAs in flounder tissues suggests, however, that IGF-I is essentially involved in skeletal growth of the teleost.

In conclusion, we have demonstrated the existence of two distinct IRs (fIR-1 and fIR-2) and two distinct IGF-IRs (fIGF-IR-1 and fIGF-IR-2) in the flounder. Northern blot analyses revealed that the major transcripts for fIR-1, fIR-2, fIGF-IR-1 and fIGF-IR-2 were approximately 13, 8, 9 and 11 kb respectively. RNase protection assays showed that the fIR and fIGF-IR transcripts were present in a wide range of tissues, with the highest levels in the heart.

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