Newly-identified receptors for peptide histidine-isoleucine and GHRH-like peptide in zebrafish help to elucidate the mammalian secretin superfamily

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Abstract

A group of ten hormones in humans are structurally related and known as the secretin superfamily. These hormones bind to G-protein-coupled receptors that activate the cAMP pathway and are clustered as the secretin or B family. We used an evolutionary approach with zebrafish as a model to understand why some of these hormones, such as peptide histidine-methionine (PHM) and pituitary adenylate cyclase-activating polypeptide (PACAP)-related peptide (PRP) in humans lack a receptor. We used molecular techniques to clone two full-length receptor cDNAs in zebrafish, which were analyzed for amino acid sequence and ligand-binding motifs, phylogenetic position, synteny, tissue expression, functional response, and signaling pathway. Evidence is provided that the two cDNAs encoded the peptide histidine-isoleucine (PHI) receptor and PRP receptor, which is known as GHRH-like peptide (GHRH-LP) receptor in non-mammals. Further, we cloned a zebrafish cDNA encoding the peptides PHI and vasoactive intestinal peptide (VIP). The PHIR had been previously labeled as one type of a VIP-PACAP (VPAC2R) shared receptor based only on sequence data. The PHIR cDNA, transfected into COS7 cells, responded to zebrafish PHI in a sensitive and dose-dependent manner ($EC_{50} = 1.8 \times 10^{-9}$ M) but not to PACAP and VIP. The GHRH-LP receptor responded to both zebrafish GHRH-LP1 and GHRH with a 3.5-fold greater response to the former. For comparison, two zebrafish receptors (PAC1R and VPAC1R) and two human receptors (VPAC2R and GHRHR) were tested with human and/or zebrafish peptides. Unexpectedly, zebrafish VIP activated its PAC1R suggesting that in evolution, PAC1R is not always a specific receptor for PACAP. We conclude that zebrafish, like goldfish, have a specific receptor for PHI and GHRH-LP. Our evidence that zebrafish PHI is more potent than human PHM in activating the human VPAC2R ($EC_{50} = 7.4 \times 10^{-9}$ M) supports our suggestion that the VPAC2R and PHIR shared a common ancestral receptor.

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Introduction

In humans, the secretin superfamily has ten hormones and at least nine receptors, all of which are listed in Table 1 with abbreviations and gene names. To date, one of the hormones lacks a specific (high-affinity) receptor and several other hormones share receptors. Only three of the ten hormones are encoded in a single gene, whereas the other hormones are encoded as two or three hormones within a gene. One human gene encodes both pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP-related peptide (PRP). Another human gene encodes both vasoactive intestinal polypeptide (VIP) and peptide histidine-methionine (PHM), whereas a third gene encodes glucagon and glucagon-like peptides 1 and 2. The remaining three genes each encode only a single hormone: growth hormone-releasing hormone (GHRH), secretin (SCT), or glucose-dependent insulinotropic polypeptide (GIP). The nine receptors that

bind these hormones are also structurally related as seven transmembrane receptors, which are grouped within the type B (or type 2) family of G-protein-coupled receptors (Sherwood *et al.* 2000, Ulloa-Aguirre & Conn 2000, Cardoso *et al.* 2005) and named by the International Union of Pharmacology (Foord *et al.* 2005). An understanding of the evolutionary history of the secretin superfamily of hormones and receptors may help to explain the gene organization, receptor binding, and function of this group of hormones.

We selected zebrafish as a comparison with humans to probe several critical questions: 1) why do peptide histidine-isoleucine (PHI; non-human) and PHM (human) lack a specific receptor with high-affinity binding, 2) why does VIP lack a specific receptor, 3) why are there two shared receptors (VPAC1R and VPAC2R) that are activated equally by both PACAP and VIP, 4) why does PRP not have a receptor, and 5) is there a full complement of secretin superfamily hormones and receptors in zebrafish? An examination of these

Table 1 The secretin superfamily of hormones and receptors in zebrafish and humans

	Abbreviations		Zf-1	Zf-2	Human	Human gene
Peptide						
Pituitary adenylate cyclase-activating polypeptide	PACAP		++	++	++	<i>ADCYAP</i>
GH-releasing hormone-like peptide	GHRH-LP	(PRP)	++	++	++	<i>ADCYAP</i>
Vasoactive intestinal polypeptide	VIP ^a		++	++	++	<i>VIP</i>
Peptide histidine-isoleucine/methionine	PHI ^a /PHM		++	++	++	<i>VIP</i>
Glucagon	GLUC		++	++	++	<i>GCG</i>
Glucagon-like peptide 1	GLP1		++	++	++	<i>GCG</i>
Glucagon-like peptide 2	GLP2		++	—	++	<i>GCG</i>
GH-releasing hormone	GHRH		++	—	++	<i>GHRH</i>
Secretin	SCT		—	—	++	<i>SCT</i>
Glucose-dependent insulinotropic polypeptide	GIP		++	—	++	<i>GIP</i>
Receptor						
Pituitary adenylate cyclase-activating polypeptide	PAC1R		++	+	++	<i>ADCYAP1R1</i>
GH-releasing hormone-like peptide	GHRH-LPR ^a	(PRPR ^a)	++	+	—	
VIP–PACAP receptor 1	VPAC1R		++	+	++	<i>VIPR1</i>
VIP–PACAP receptor 2	VPAC2R		—	—	++	<i>VIPR2</i>
Peptide histidine-isoleucine	PHIR*		++	—	—	
Glucagon	GLUC-R		++	—	++	<i>GCCR</i>
Glucagon-like peptide 1	GLP1R		++	—	++	<i>GLP1R</i>
Glucagon-like peptide 2	GLP2R		+	—	++	<i>GLP2R</i>
GH-releasing hormone	GHRHR		++	—	++	<i>GHRHR</i>
Secretin	SCTR		—	—	++	<i>SCTR</i>
Glucose-dependent insulinotropic polypeptide	GIPR		+	—	++	<i>GIPR</i>

⁺⁺mRNA transcribed; ⁺peptide or receptor identified in genome but not isolated; Alternate names include GHRH-LP in non-mammals but PACAP-related peptide or PRP in mammals; PHI in most vertebrates is PHM in humans; references and structures are at <http://www.ncbi.nlm.nih.gov>.
^aIdentified in zebrafish in the present paper.

hormones and receptors in zebrafish is of considerable interest, as this fish is a well-known model for developmental and endocrine studies (McGonnell & Fowkes 2006).

Of the ten hormones in humans, PACAP is the most conserved member. The two biologically active forms, PACAP₂₇ and PACAP₃₈, are identical as the latter is a C-terminal extension of PACAP₂₇ (Miyata *et al.* 1989, 1990). Zebrafish also have PACAP₂₇ and PACAP₃₈, but the gene encoding the peptides has been duplicated producing two forms of each peptide (Fradinger & Sherwood 2000, Wang *et al.* 2003). PACAP has a wide range of functions as a neuromodulator, neurotrophin, smooth muscle relaxant, and a releasor of pituitary hormones (Sherwood *et al.* 2000, Chow *et al.* 2003). In fish, PACAP additionally can release growth hormone (Parker *et al.* 1997, Wong *et al.* 1998). The receptors that activate these physiological actions are PAC1R, which preferentially binds PACAP with high affinity compared with VIP, and VPAC1R that shows similar potency with PACAP or VIP (Laburthe *et al.* 2003). Both PAC1R and VPAC1R are identified in humans and zebrafish, but humans also have a VPAC2R that is related in structure and function to VPAC1R. Fish have receptors that resemble VPAC2Rs in structure, but they have not been tested for binding to any peptides.

Although PRP and PACAP share the same precursor, the function of PRP in mammals is unknown. In human, PRP is 52% identical in amino acids to GHRH₂₇,

whereas in zebrafish one of the two PRP peptides, known as GHRH-like peptide1 (GHRH-LP1) has a higher identity (59%) with the zebrafish true GHRH₂₇. Also, release of GH has been observed in some fish experiments but not in others after application of GHRH-LP *in vivo* or *in vitro* (see Tam *et al.* 2007 for review). A PRP receptor has not been identified for any mammal but a full-length sequence (PRPR or alternatively GHRH-LPR) in goldfish (Chan *et al.* 1998) and partial sequence (transmembrane domains 5–7) in zebrafish (Fradinger *et al.* 2005) have been reported.

Another two neuropeptides, PHM and VIP, are listed in Table 1; they are cleaved from a single precursor resulting in PHM₂₇ and VIP₂₈ in humans with 44% amino acid identity. VIP is a potent peptide that mediates smooth muscle relaxation, stimulates endocrine and exocrine secretion, regulates circadian rhythms and neuromodulates, whereas PHM is thought to be a less potent version of VIP that uses the same receptors, VPAC1R and VPAC2R. The name of PHM in human becomes PHI in other vertebrates, as the C-terminal peptide is isoleucine (I) rather than methionine (M). In zebrafish, a PHI/VIP cDNA has not been cloned. The receptors for VIP are shared with PACAP as noted earlier. However, a receptor for PHM/PHI has not been identified for any mammal and only one PHIR (goldfish) has been characterized to date (Tse *et al.* 2002).

Mammalian GHRHs act on the pituitary to release GH. A 'true' GHRH cDNA was identified only in 2007 for chicken (Wang *et al.* 2007), goldfish, and zebrafish (Lee *et al.* 2007), and shown to be a homolog with the mammalian form. Relevant here, the zebrafish and goldfish GHRH receptors have been shown to be homologs of the mammalian receptor using synteny and response to fish true GHRH *in vivo* and *in vitro* (Lee *et al.* 2007).

In the present experiments, our aim was to use zebrafish to identify a PHIR and determine which signaling pathway is coupled to this receptor. To test the biological activity of the PHIR, we cloned the PHI/VIP cDNA precursor and synthesized the two peptides. The zebrafish peptides of PHI and VIP along with zebrafish PACAP1 and 2 were used to test the signaling path of the zebrafish PHIR. To further expand our understanding of the evolution of the secretin superfamily, we also cloned and expressed GHRH-LP receptor and characterized its signaling properties. Human and zebrafish peptides were tested also on human VPAC2R and GHRHR. Our hypothesis is that the evolution of the secretin superfamilies of hormones and receptors in vertebrates has resulted in the loss or alteration of certain receptors in mammals with subsequent changes in ligand binding. Phylogenetic analysis and synteny maps were used to evaluate the evolution of the receptors.

Materials and methods

Animals

Wild-type zebrafish (*Danio rerio*) were purchased from a local pet store and maintained in our laboratory with a 14h light:10h darkness cycle at 28.5 °C. Procedures were approved by the Animal Care Committee at the University of Victoria. The tissues of adult zebrafish were dissected and frozen in liquid nitrogen.

Peptides

The sequences of seven zebrafish peptides used to test receptor activity are shown in Table 2. All peptides were

synthesized at the Salk Institute (La Jolla, CA, USA) using a CS-biopeptide synthesizer (model CS536, CS Bio Co. Inc., San Carlos, CA, USA) on a methyl benzhydrylamine resin using Boc-strategy. The peptides were cleaved with hydrofluoric acid, concomitantly deprotected, and then purified as described in Adams *et al.* (2003). The purity of the peptides was characterized by capillary zone electrophoresis performed on a Beckman P/ACE System 2050 connected to a ChromJet integrator (Spectra Physics, San Jose, CA, USA). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of the peptides was measured on an ABI Perseptive DE-STR instrument (PE Applied Biosystems, Foster City, CA, USA). In addition, three human peptides were tested: PHM₂₇ and VIP₂₈ (Bachem, Torrance, CA, USA), and GHRH₄₄ (Salk Institute).

Total RNA isolation and cDNA synthesis

Total RNA was extracted from 11 tissues of adult zebrafish: brain, eye, heart, testis, ovary, spleen, kidney, gill, skin, gut, and swimbladder according to the manufacturer's protocol (RNeasy Mini Kit, Qiagen Inc.) The concentration of total RNA was measured and 900 ng total RNA from each sample was reverse transcribed with Oligo (dT) and Superscript II (Invitrogen) according to the manufacturer's instructions.

RACE reactions for zebrafish *phir*, *ghrh-lpr*, and *vip* (PHI/VIP)

Total RNA (500–600 ng) from adult zebrafish brains was used for the synthesis of cDNA with the First Choice RLM-RACE kit (Ambion) following the manufacturer's instructions. The 5' and 3' RACE primers for *phir* were designed from a published partial sequence for *vipr2* (VPAC2R) (Wang *et al.* 2003) and *ghrh-lpr* (Fradinger *et al.* 2005). A partial predicted zebrafish VIP peptide sequence was found by performing a BLAST search with VIP of rat/mouse, guinea pig, chick, frog, trout/bowfin, cod, dogfish in the Vega Multi Blast View program (http://vega.sanger.ac.uk/Multi/blastview?species=D._rerio); this partial

Table 2 Amino acid sequences of the zebrafish peptides tested in the receptor activation assay

Peptide	Sequence	References
PACAP ₁₂₇	HSDGVFTDSYSRYRKQMAVKKYLATVL-NH ₂	Fradinger <i>et al.</i> (2000)
PACAP ₂₇	HSDGIFTDIYSRYRKQMAVKKYLAAYL-NH ₂	Wang <i>et al.</i> (2003)
VIP ₂₈	HSDAIFTDNYSRFRKQMAVKKYLNLSVL-NH ₂	(Present results)
PHI ₂₇	HADGIFTSGYSKLLGQLSARRYLESIL-NH ₂	(Present results)
GHRH-LP ₁₂₈	HADGMFNKAYRKALGQLSARKYLHTLMA	Fradinger <i>et al.</i> (2000)
GHRH-LP ₂₉	HADGLLDRLALDILVQLSARKYLHSLMAV	Wang <i>et al.</i> (2003)
GHRH ₂₇	HADAIFTNSYRKVLGQISARKFLQTVM-NH ₂	Lee <i>et al.</i> (2007) and Wang <i>et al.</i> (2007)

predicted zebrafish VIP peptide was identical to trout VIP. Primers for 3' RACE of *vip* (PHI/VIP) were designed to this predicted nucleotide sequence. Later, 5' RACE primers were designed from our 3'RACE PCR sequence. RACE-PCRs were carried out for 35 cycles at 94 °C for 30 s, 55 °C annealing temperature for 30 s, and 72 °C for 1 min with a 7-min extension at 72 °C on the last cycle. All primers are listed in Table 3. The PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with

ethidium bromide. Then, PCR products were selected and isolated with QIAquick Gel Extraction Kit (Qiagen), and ligated into PGEM-T vector (Promega Corp.)

Data mining, phylogenetic analysis, and synteny comparisons

The NCBI database was used to search for PAC1R, VPAC1R, VPAC2R, GHRHR and GHRH-LPR in human,

Table 3 Primers used to amplify cDNA in zebrafish

Primers	Sequence (5'–3')	Target
RACE		
<i>phir</i>		
R ^a 274a	CAGGTTCAGATGGATGTAGT	5' cDNA
R267b	AGATGGATGTAGTTCCTGGTG	5' cDNA
F ^b 3b	GTCCTGCTGCCCTCAAAA	3' cDNA
F191	CTCATCGCCCTCATCACC	3' cDNA
<i>ghrh-lpr</i>		
R-615	TCCAGAACGCCAGTCGTCATCAA	5' cDNA
R-190	CCCCGACTCCAGGACTTTTC	5' cDNA
<i>vip</i> (PHI/VIP)		
R706	AATAAAGATTTCCAATGCCCGACCC	5' cDNA
R584	TCATCGTCCAATCAGCAGTC	5' cDNA
F260	GTAGTGATGATTTGATGGAAGACC	3' cDNA
Clones for functional study		cDNA
<i>phir</i>		
F1	AAAGTACTGCAAACCGCACATGAT	
R2096	CTTACAATCACCCTTCACA	
F172NheI	TGCTAGCGCCACCATGGACATGAGCCTCGTCAACT	
R1551XbaI	ATTCTAGAAGGGCAGTGAAGAGCGAGCG	
<i>ghrh-lpr</i>		
F5	AAGTGAATCTGACTGTGCG	
R1675	ATCATATATATTTAAATCATTCTTATTTCC	
F120NheI	TTGCTAGCGCCACCATGGCGTTGTACATCTGTGCT	
R1436XhoI	TTCTCGAGTACAATCCAGTGGCGAGTTCA	
Tissue expression primers		cDNA
<i>phir</i>		
F429	CAGAGATGGGATCATTAGCAG	
R853	GTAAGTACAATCCTTCAACCAG	
<i>ghrh-lpr</i>		
F79	GTCGATGAGAAAGAGCGGCTGT	
R701	GAAGAAACATCCTCAAGACAATACTG	
<i>ghrhr</i>		
F30	CCGTGTGCGCGTGGACTTTT	
R625	CAGGCCACAGTAGACAGAGAGCAG	
<i>vip</i> (PHI/VIP)		
F41	CAGGTGTCCACGGCTGTGTG	
R596	AGGGCTCCATCGAAACGCAG	
<i>vipr1</i> (VPAC1R)		
F66	AACTTGCTATGTTTTACTCGTCTG	
R537	ATCGTCAACAGTGCCATTCA	
<i>adcyap1r1</i> (PAC1R)		
F1123	GCCCTGTGGTGGCATCAATC	
R1613	TCAGGTTGGCAGGTTGAGGT	
<i>β-actin1</i>		
F19	ATTGCCGCACTGGTTGTT	
R1033	CTCCGATCCAGACAGAGTATTTT	

The enzyme restriction sites are underlined.
^aF, forward primer.
^bR, reverse primer.

chicken, frog, goldfish, fugu, and zebrafish genomes. The amino acid sequence of the open reading frame of zebrafish PHI receptor with its seven transmembrane domains was used separately to search for homologous receptors in the same genomes as above and in those of medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), *Tetraodon nigroviridis*, and salmonids (Salmonidae family) not only in NCBI website (www.ncbi.nlm.nih.gov) but also in Ensembl (www.ensembl.org) and the Salmon Genome Project database (www.salmongenome.no). The deduced amino acid sequences were aligned using the ClustalW alignment program (Thompson *et al.* 1994) with BLOSUM62. A neighbor-joining (NJ) phylogenetic tree was generated based on the ClustalW alignment of the amino acids from transmembrane domains 1-7 by MEGA3.0 (Kumar *et al.* 2004) to produce the tree using JTT (Jones–Taylor–Thornton) substitution. In addition, a maximum-likelihood (ML) tree was generated with 100 bootstraps and the JTT substitution using PHYML (Guindon & Gascuel 2003) and its online web server (<http://atgc.lirmm.fr/phyml/menu.html>; Guindon *et al.* 2005).

The GenBank accession numbers for the receptors isolated here are zebrafish PHIR (EU150381) and zebrafish GHRH-LPR (EU150382). For other sequences, the numbers are: human (*Homo sapiens*) GHRHR (NP000814); chicken GHRHR (ABB84385); goldfish GHRHR (ABJ55978); zebrafish GHRHR (ABJ55981); chicken PRP receptor (PRPR; CAC82589); goldfish GHRH-LPR (AAC15698); fugu GHRH-LPR (CAC82589); human VPAC2R (P41587); chicken VPAC2R (NP_001014970); fugu VPAC2R (AJ408877); fugu VPAC2RB (AJ296143); trout (AY706217); human VPAC1R (P32241); marsh frog VPAC1R (Q9YHC6); goldfish VPAC1R (AAB05459); zebrafish VPAC1R (NP_001013371.1 and for the second receptor ENSDARP00000046126); fugu VPAC1RA (CAC82588); fugu VPAC1RB (CAC82587); human PAC1R (P41586); goldfish PAC1R (O73769); zebrafish PAC1R-short (AAW65134.1 and for the second receptor XP682980); fugu PAC1RA (CAD35690); and fugu PAC1RB (CAD33842). The Ensembl accession numbers are: medaka VPAC2RA (ENSORLP00000023740), medaka VPAC2RB (ENSORLP0000007394), *Tetraodon* VPAC1R (GSTENP00016553001), stickleback VPAC2RA (ENSGACP0000002397), and stickleback VPAC2RB (ENSGACP00000023187).

Ensembl also lists two accession numbers for zebrafish VPAC2R, one of which (ENSDARG00000012353) is the full-length (441 amino acids) receptor that we call the PHIR and the other (ENSDARG00000067568) is a truncated (318 amino acids) receptor; the shorter receptor lacks an extracellular N-terminal domain and its 318 amino acids are 100% identical to the longer receptor. The truncated receptor is not considered further. The duplicate receptor for zebrafish VPAC1R

was not included in the ML or NJ trees as the receptor had low sequence similarity in TMD 3–6, probably due to inaccurate exon predictions. However, we have included in the ML tree a second zebrafish GHRH-LPR (ENSDARP00000070262), which lacks 100 amino acids in the N-terminal domain, because the remaining amino acids include all 7 TM regions and are distinct from the first form of the receptor; the truncated receptor has not been cloned to date but the genomic sequence shows a 26 amino acid insertion compared with other GHRH-LPRs (Fig. 2). The zebrafish glucagon receptor (GLUCR) (XM_685886) was used as the outgroup for tree construction. Goldfish PHIR was reported earlier but does not have an accession number (Tse *et al.* 2002). Two receptors with accession numbers are not considered further as second forms in our study, as they are almost identical (99–99.5%) to ones listed in Fig. 1 or 2; one of these receptors is a second form of fugu GHRH-LPR (ENSTRUP00000032622) and the other is a second form of trout VPAC2R (CU069615), which is only a fragment (residues 255–440) of the full-length receptor.

To examine the syntenic arrangement of genes surrounding the PHI gene and VPAC2R gene, a sequence similarity approach was used. All data were obtained from the Ensembl database. The neighboring genes of the two receptors were used to search for orthologs in zebrafish, medaka, chicken, and human genomes using the MultiContigView and then selecting 'View alongside' and the species of interest. Also, the same genes were searched in Ensembl Blast View and NCBI database to avoid missing any data. The medaka VPAC2R genes were not labeled A or B in Ensembl so we assigned A to the receptor that clustered with other fish VPAC2Rs in the ML tree (based on comparison of the seven transmembrane regions). However, medaka VPAC2RB was closer to zebrafish PHIR in a comparison of total length (Table 4). Therefore, we used the medaka B receptor for synteny comparisons; also the gene neighbors matched those of the zebrafish receptor and the medaka A receptor could not be compared, as it was on the end of a scaffold.

Tissue distribution of zebrafish mRNA: *phir*, *adcyap1r1* (PAC1R), *vipr1* (VPAC1R), *ghrh-lpr*, *vip* (PHI/VIP)

Tissue-specific distribution of each mRNA was performed by RT-PCR on 11 tissues of zebrafish. Primers for the *adcyap1r1*-short isoform, *vipr1*, and *phir* were designed according to the NCBI Gene Bank database (accession No.: AAW65134.1; NP_001013371.1; NP_571854). *ghrh-lpr* and *vip* primers were designed according to our 5' and 3' RACE sequences. A control reaction was prepared using 1 µl of cDNA amplified with zebrafish actin primers. All PCRs were carried out on 1 µl cDNA for 35 cycles at different annealing temperatures: 58 °C

Table 4 Percentage of amino acid identity of aligned zebrafish peptide histidine-isoleucine (PHI) receptor with others in the same gene family

	1	2	3	4	5	6	7	8	9	10	11	12
1 Zebrafish PHIR		80	53·9	61·1	64·6	51·1	49·8	60·2	46·9	57·5	58·8	55·5
2. Goldfish PHIR			52·4	56·5	59·5	46·9	49·5	56·9	45·9	54·7	56	51·2
3. Fugu VPAC2RA ^a				51·7	58·2	69·6	65·5	53·6	66·1	49·1	48·6	46·5
4. Fugu VPAC2RB ^a					56·1	48·4	49·5	76·7	46·3	72·2	57·7	55·7
5. Trout VPAC2R						53	49·7	56·5	50·4	53	53·7	52·9
6. <i>Tetraodon</i> VPAC2R ^a							54·1	50·8	54·5	48	43·2	42·2
7. Stickleback VPAC2RA ^a								49·2	54	45	43·8	41·9
8. Stickleback VPAC2RB ^a									47·2	81	57·7	56·1
9. Medaka VPAC2RA ^a										44·4	42·3	41·5
10. Medaka VPAC2RB ^a											54·8	53
11. Chicken VPAC2R												72·9
12. Human VPAC2R												

Amino acid identity was determined by using the ClustalW alignment program with BLOSUM62.
^aMeans receptor amino acid sequence is partial as shown in Fig. 1.

(*bactin1*), 60 °C (*adcyap1r1*, *phir*), 62 °C (*vip*, *vipr1*, *ghrh-lpr*, *ghrhr*). All PCR products spanned at least two exons as a control check for potential genomic contamination. PCR products of the expected size were sequenced and confirmed.

Transfection and expression of zebrafish PHIR, PAC1R-short, VPAC1R, GHRH-LPR and human VPAC2R and GHRHR

Constructs of cDNA full-length coding sequences for receptors PAC1R-short and VPAC1R were a gift from Javier Tello as previously reported (Fradinger *et al.* 2005). The cDNAs for PHIR and GHRH-LPR containing full-length open reading frames with flanking restriction enzymes sites and a Kozak sequence were subcloned into pcDNA3.1 (–) (Invitrogen). Two rounds of PCRs were conducted with platinum Taq polymerase high fidelity (Invitrogen) as outlined by the manufacturer. Primers are listed in Table 3. Human vasoactive intestinal peptide receptor 2 (VPAC2R) and GHRHR cDNA clones were obtained from University of Missouri-Rolla (UMR) cDNA Resource Center (Clone ID: VPAC2R00000, GHRHR00000; GB Acc. No.: NM_003382, AY557192). All six receptors were separately transfected into COS7 cells (American Type Culture Collection, Manassas, VA, USA) as described (Tello *et al.* 2005) with a few modifications: 1) cells were plated at a density of 60 000 cells/well, 2) medium was replaced after 14 h of transfection with fresh VP-SFM (Invitrogen) for later cAMP assay or replaced with labeling medium (Medium 199, Invitrogen) plus 0·3% bovine albumin (Sigma–Aldrich) for the inositol phosphate (IP) assay, and 3) labeling medium with isobutylmethylxanthine (IBMX) and 10 mM LiCl was used to incubate cells during ligand stimulation. The cAMP assay was done with an Amersham cAMP enzyme immunoassay Biotrak system

(GE Healthcare, Little Chalfont Buckinghamshire, UK). In the total IP accumulation assay, quantitation was performed with cell extracts by the multi-well filtration method (Chengalvala *et al.* 1999).

Data analysis

All IP and cAMP samples were measured in triplicate within each assay and each experiment was independently repeated at least thrice. Data analysis was performed using PRISM3 software (GraphPad Software Inc., San Diego, CA, USA) with nonlinear regression (curve fit) and sigmoidal dose–response curves. The EC₅₀ values (dose of peptide stimulating half-maximal IP or cAMP response) of each peptide were calculated from the means of at least three independent experiments. The data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. *P* < 0·05 was considered as statistically significant.

Results

Isolation and structural analysis of zebrafish PHIR, GHRH-LPR, and PHI/VIP cDNAs

Full-length cDNAs obtained with the use of total RNA from zebrafish brains and RACE reactions were sequenced in both the forward and reverse directions. The zebrafish PHIR cDNA was 2088 bp in length (GenBank accession No. EU150381) with an open reading frame of 1323 bp (from 169–1491) encoding a 441 amino acid protein (Fig. 1A and B). A Kyte–Doolittle hydrophobicity plot (data not shown) of the deduced protein revealed that it has seven transmembrane domains. Comparison of the zebrafish PHIR deduced amino acid sequence of the full receptor revealed that its

Figure 1 (continued)

		TMD1			TMD2		
		160	170	180	190	200	
Zebrafish PHIR		TLGHSLSLIA	LITGSTILCL	FR-KLHCTRN	YIHLNLFSSF	ILRAIAVLVK	
Goldfish PHIR		TLGHSLSLIA	LITGSTILCL	FR-KLHCTRN	YIHLNLFSSF	ILRAIAVLVK	
Fugu VPAC2RA		TLGHSLSLVT	LLTSTFIMCF	FR-RLHCTRN	YIHLNLFVSF	MLRAVAVLAK	
Fugu VPAC2RB		TLGHSLSLIA	LITGSAVLCL	FR-KLHCTRN	YIHLNLFLSF	ILRAVAVLAK	
Trout VPAC2R		TLGHSLSLIA	LITSTLIILCL	FR-RLHCTRN	YIHVNLIVSF	ILRAVAVLVK	
Tetraodon VPAC2R		TLGYSLSLVA	LLASTFIICF	FR-RLHCTRN	YIHLNLFVSF	MLRAVAVLAK	
Stickleback VPAC2RA		TLGHSLTFIT	LLTSTVILCL	YR-KLHCTRN	YVHLNLFLSF	MLRAAAVLAK	
Stickleback VPAC2RB		TLGHILSLIA	LTTGSAILCL	FR-KLHCTRN	YIHLNLFSSF	VLRAVAVLAK	
Medaka VPAC2RA		-VSNTSTLSF	FLEMSFKSCF	LK-RLHCTRN	YIHVNLFVSF	MLRAVAVLAK	
Medaka VPAC2RB		TLGHSLSLIA	LITGSAVLCL	FR-KLHCTRN	YIHLNLFVSF	ILRAVAVLAK	
Chicken VPAC2R		TLGHSVSLIA	LTTGSIIILCL	FR-KLHCTRN	YIHLNLFLSF	ILRAISVLVK	
Human VPAC2R		TLGYSVSLMS	LATGSIIILCL	FR-KLHCTRN	YIHLNLFLSF	ILRAISVLVK	
Clustal consensus		.. :: : : *	: : : : *	: : : : *	: : : : *	: : : : *	

		TMD3			TMD4		
		210	220	230	240	250	
Zebrafish PHIR		DAILFS-HEN	GECTVQP-SL	M-----	--GCKVSLVI	LNIFIMANFY	
Goldfish PHIR		DAILFS-HGN	VECSKHP-SL	VNESIHPEYL	QIGCKASLII	FHYFIANFY	
Fugu VPAC2RA		DTLLFSDDET	TDCSTQP-SL	V-----	--GCKAILVF	FNIFVMANFF	
Fugu VPAC2RB		DDILFN--RT	SQCSNQP-SL	AG-----	---CKTSLVF	FQYFIMANFF	
Trout VPAC2R		DSVIFSHNEN	TDCSTQP-SL	VG-----	---CKVILVM	FNIFVMANFF	
Tetraodon VPAC2R		DALLFSDDET	TDCSTQP-SL	M-----	--GCKAVMVL	FNIFVMANFF	
Stickleback VPAC2RA		DTLLFSGEQN	AECQAQP-SL	VRS-----	--GCKVVLVF	FNIFVMA-TF	
Stickleback VPAC2RB		DDILFN--RT	SQCSNQP-SL	AG-----	---CKASLVF	FQYFIMANFF	
Medaka VPAC2RA		DTLLFSDEDA	TDCSTQP-SL	V-----	--GCKATLVF	FNIFVMANFF	
Medaka VPAC2RB		DDILFN--KT	LQCSNQS-SL	LG-----	---CKASLVF	FQYFIMANFF	
Chicken VPAC2R		DDILYSSSNT	AHCPEDQTSW	VG-----	---CKAILVF	FQYGVMANFY	
Human VPAC2R		DDVLYSSSGT	LHCPDQPSW	VG-----	---CKLSLVF	LQYCIMANFF	
Clustal consensus		* : : .	. * . *		** : : :	: : * : *	

		TMD5					
		260	270	280	290	300	
Zebrafish PHIR		WLLVEGLYLH	TLLMVIFSEN	RHFIIYLLIG	WGFPVVFVTP	WIVCRIYLED	
Goldfish PHIR		WLLVEGLYLH	TLLMVIFSEN	RHFIIYLLIG	WGFPVVFMI	WIVCRIYLED	
Fugu VPAC2RA		WLLVEGLYLH	TLLLIIRNAS	IRLPVYMLIG	WGIPFVFMVA	WVICRVNLED	
Fugu VPAC2RB		WLLVEGLYLH	TLLVVFIFSEN	RHFIVYLFIF	WGIPAVFVFV	WVMRIYLED	
Trout VPAC2R		WLLVEGLYLH	TLLLVITYAY	THLAVYLTIG	WGLPSVFLV	WVFCRIYLED	
Tetraodon VPAC2R		WLLVEGLYLH	TLLLIIRHSS	IRLSVYMLIG	WGIPFVFTLA	WVICRINLED	
Stickleback VPAC2RA		LAAVEGLYLT	RYRESERSSS	CRVA-----	-GIPLVFMAA	WIACRINLED	
Stickleback VPAC2RB		WLLVEALYLH	TLLVVFIFSEN	RHFVVYMFIF	WGIPVVFVSA	WVMTRIYLED	
Medaka VPAC2RA		WLLVEGLYLH	TLLLVHITYS	TRLSIYMFIF	WGIPFVFVVA	WISRVNLED	
Medaka VPAC2RB		WLLVEGLYLH	TLLVVFIFSEN	RHFIIYMFIF	WGIPVVFVSA	WVITRIYLED	
Chicken VPAC2R		WLLVEGLYLH	ILLVLIFSPN	RHFTVYLLIG	WGIPTFIIFT	WTVTRIILED	
Human VPAC2R		WLLVEGLYLH	TLLVAMLPPR	RCFLAYLLIG	WGLPTVCIGA	WTAARLYLED	
Clustal consensus		** . **	.		** :	* : * :	

		TMD5					
		310	320	330	340	350	
Zebrafish PHIR		TGCWERIDNP	IPWRVINWPI	MASVILNFIL	FISIIRIIVQ	KLRCPDVGN	
Goldfish PHIR		TGCWERNDAP	IPWRVINWPI	MASVILNFIL	FISIIRIIVQ	KLRCPDVGN	
Fugu VPAC2RA		TRCWEMNENP	VPNRLINWPI	MASVIINFIF	FISIIRIIVQ	KLQCADVGN	
Fugu VPAC2RB		TGCWETNDNP	IPNWVINGPI	GFSIMVNFLL	FVSIIRIIVQ	KLRCPDVGN	
Trout VPAC2R		TGCWERNDIP	TPWRVINWPI	MASVIINFVL	FISITRIIVQ	KLRCSDVGN	
Tetraodon VPAC2R		TGCWEKHENP	VLSRLIHWPI	MASVIINFIL	FISIIRIIVQ	KLRCIDVGN	
Stickleback VPAC2RA		TQCWEMNENP	IANRLMNWPI	MASVIMNFVL	FVSIIRIIVQ	KLRCPDVGN	
Stickleback VPAC2RB		TGCWERNNDP	IPNWVINGPI	GFSIMVNFIL	FISIIRIIVQ	KLRCPDVGN	
Medaka VPAC2RA		TSCWEINDNP	VPDRLINWPI	MASIIINFIL	FISIIRIIVQ	KLRCSEVGN	
Medaka VPAC2RB		TGCATTSDD--	----ICKSSI	WFFIRVNFIL	FISIIRIIVQ	KLRCPDVGN	
Chicken VPAC2R		TGCWDTNEHG	GPWWVIRIPI	LISIIVNFIL	FISIIRIILQ	KLRCSDVGN	
Human VPAC2R		TGCWDTNDNS	VPWWVIRIPI	LISIIVNFVL	FISIIRIILQ	KLRCSDVGN	
Clustal consensus		* *	:	: . *	: : * :	* : * : * : *	

Figure 1 (continued)

Figure 1 (A) Predicted structure of zebrafish PHIR gene from zebrafish genome database in Vega. The gene size (> 79 kb) is indicated on the right of the gene. Exons are numbered and are shown as closed boxes; a solid horizontal black line indicates introns. The distance between the first five exons is indicated by a number above the intron line. (B) ClustalW alignment of amino acids of zebrafish PHIR with other known PHIR and VPAC2Rs. The transmembrane domains are annotated above the sequence. Thirteen conserved cysteines are marked with a black dot, although fugu VPAC2RB, stickleback VPAC2RA, and medaka VPAC2RA each lack one cysteine. Two potential conserved N-glycosylation sites (Asn-X-Thr/Ser) in the N-terminal extracellular domain for zebrafish are labeled with a black triangle. Opened circles or ovals show where Asn is not conserved. All signature motifs for both PHIRs and VPAC2Rs are in open boxes.

highest match is with goldfish PHIR. Also, the PHIR shares high sequence identity with trout VPAC2R (65%), other teleost VPAC2Rs (47–61%), chicken VPAC2R (59%), and human VPAC2R (56%; [Table 4](#)). For other members of the secretin receptor family, zebrafish PHIR shares

sequence identity with zebrafish PAC1R-short (42%), VPAC1R (42%), GHRH-LPR (37%), and GHRHR (35%). The percent identity plus the functional study suggests that the present cDNA encodes VPAC2R a PHIR, not a VPAC2R. Hereafter, we refer to the receptor as PHIR.

The full-length cDNA of the PHIR blasted against the zebrafish genome database (Vega Multi Blast View) shows that the zebrafish PHIR gene spans more than 79kb on chromosome 24 and consists of 14 exons (Fig. 1A). Sequence alignment of PHIRs and VPAC2Rs from various species (Fig. 1B) indicates that zebrafish PHIR has amino acid signature motifs such as PDV and IIRIL characteristic of VIP binding receptors, and has RLAK between TMD5 and TMD6 for coupling to G_s

(Chow *et al.* 1997, Cardoso *et al.* 2004). Another amino acid motif, the highly conserved region for the mammalian secretin B family members at TMD7, FQGBBVXXBYCFXNXEXQ (Lok *et al.* 1994), was identified where X represents any amino acid residue and B represents any hydrophobic amino acid residue (Fig. 1B). In addition to these motifs, two conserved residues, R and K, localized within TMD2 and associated with VPAC1R activation (Solano *et al.* 2001)

Figure 2 (continued)

Figure 2 ClustalW alignment of amino acids for two zebrafish (zf) GHRH-LPRs (*the truncated form) with goldfish (gf), fugu (fu), and chicken (ch) GHRH-LPRs (a.k.a PACAP-related peptide receptor or PRPR). The transmembrane domains are annotated above the sequence. Thirteen conserved cysteines in the fish receptors are marked with black dots and three potential conserved *N*-glycosylation sites in the N-terminal extracellular domain in fish are labeled with a black triangle. Signature motif 'RLAK' is shown by an open box. As noted, chicken GHRH-LPR has only three conserved cysteines and one glycosylation site in the N-terminus compared with the GHRH-LPR of fish.

were identified. Thirteen conserved cysteines, which are involved in tertiary structure and ligand binding, were also found in the zebrafish PHIR. There are only two conserved putative glycosylation sites in PHIRs in zebrafish at N-terminal extracellular sites in contrast to three conserved sites in those of chicken and mammals. However, goldfish PHI receptor along with the zebrafish PHIR and fugu putative VPAC2RA and VPAC2RB have only two glycosylation sites (Asn-X-Ser/Thr, where X is any amino acid except proline) in the N-terminal extracellular domain.

The zebrafish GHRH-LPR cDNA is 1747 bp in length (GenBank, accession No. EU150382) with an open reading frame of 1296 bp (from 181 to 1476) encoding a 432 amino acid protein (Fig. 2). A Kyte–Doolittle hydrophobicity plot (data not shown) of the deduced protein was used to identify seven putative transmembrane domains. The amino acid sequence of the zebrafish GHRH-LPR shares the highest level of identity with goldfish GHRH-LPR (88%), followed by fugu (64%), and chicken (43%) as seen in Table 5. On the other hand, zebrafish GHRH-LPR only shares 39–40% sequence identity with GHRHR of zebrafish, goldfish, chicken, and human. The intron and exon organization was not revealed by a BLAST search of the full-length cDNA of GHRH-LPR in the zebrafish genome database (Vega Multi Blast View) as the genome data are not complete. Sequence alignment of GHRH-LPRs from various species (Fig. 2) revealed that zebrafish GHRH-LPR is very similar and structurally related to receptors of other fish. Thirteen conserved cysteines and three putative glycosylation sites, and signature motifs like RLAK, FQGBBVXXBYCFXNXEXQ, and SQR were observed in zebrafish, fugu, and goldfish receptors.

The zebrafish PHI/VIP cDNA is 1092 bp in length (GenBank, accession no. EU150383) with an open reading frame of 459 bp (from 117 to 575) encoding a 153 amino acid precursor. The genomic database in Vega revealed that the PHI/VIP gene spans ~3584 bp on chromosome 13 and contains six exons (Fig. 3A and B). The zebrafish mature PHI peptide shares high sequence identity with goldfish PHI A (85%) and B (89%), chicken PHI (67%), and human PHM (78%) peptides (Tse *et al.* 2002; Fig. 3C). The zebrafish VIP is identical with the mature VIP peptide of trout. Both PHI and VIP in the zebrafish precursor cDNA are flanked at the C-terminus by a GKR, indicating that the peptides are amidated and have potential proteolytic cleavage sites (Fig. 3B).

Phylogenetic analysis and synteny comparisons

Phylogenetic analysis involved zebrafish PHIR and GHRH-LPR with other members of the secretin receptor superfamily. Analysis of all ClustalW multiple aligned receptors (TMD 1–7) was conducted by the ML

method and the NJ method with the GLUCR as the outgroup. The ML tree (Fig. 4) showed a similar result as the NJ tree (Supplementary Figure 1, see Supplementary data in the online version of the Journal of Endocrinology at <http://jme.endocrinology-journals.org/content/vol41/issue/5>). The analysis indicates that the five major groups of secretin superfamily receptors included the GHRHR, GHRH-LPR, PAC1R, VPAC1R, and the PHIR/VPAC2R; each group had a bootstrap value of 99–100. The identified zebrafish GHRH-LPR was in a subgroup with like receptors and the potential for grouping with GHRHRs (bootstrap value of 66 in NJ tree but only 46 in ML tree). The PHIR/VPAC2R group had three subgroups: 1) zebrafish and goldfish PHIRs were in a subgroup with five other teleost VPAC2RAs (bootstrap=100), 2) three teleost duplicate VPAC2Rs (receptor B, bootstrap=75) for fugu, medaka, and stickleback were together, and 3) chicken and human VPAC2Rs formed the third subgroup (bootstrap=96).

The chromosomal location of the PHIR gene or VPAC2R in zebrafish, medaka, chicken, and human are shown in Fig. 5. Two linked genes in the same chromosomal locations are also shown. In zebrafish, the PHIR gene is found on chromosome 24 along with two linked genes (*zmynd11* and *xr_029619.1* with the latter being similar to human *DIP2C*). The same arrangement is present for medaka on chromosome 17 and for chicken on chromosome 2. However, in human the VPAC2R gene on chromosome 7 has been separated from the linked genes on chromosome 10.

Functional analysis of PHIR compared with related zebrafish and human receptors

To evaluate whether the identified PHIR and PHI peptide are able to transduce a physiological signal, COS7L cells were separately transfected to express zebrafish PHIR, VPAC2R, PAC1R-short, and human VPAC2R. The ability of zebrafish peptides to activate adenyl cyclase and phospholipase C pathways was assessed by the cAMP and [³H] IP assay (Fig. 6). In COS7L cells expressing the PAC1 receptor, three peptides but not PHI were able to activate the cAMP and IP accumulation in a dose-dependent manner with different half-maximal response (EC₅₀) values (Fig. 6A and B). There was no significant difference ($P < 0.05$) in EC₅₀ values of PAC1R stimulation by the two different PACAP peptides in either the cAMP or IP pathway. But the EC₅₀ values of the PAC1R response to VIP were significantly higher ($P < 0.05$) than those of the PACAP peptides in either the cAMP or IP pathway. Hence, zebrafish PACAP was more potent than zebrafish VIP on the zebrafish PAC1R.

In cells expressing the VPAC1R, the same three peptides (PACAP1, PACAP2, VIP) were able to activate the cAMP pathway but PHI was not active in a

Table 5 Percentage of amino acid identity of aligned zebrafish growth hormone-releasing hormone-like peptide receptor (GHRH-LPR) with others in the same gene family

Zebrafish GHRH-LPR	Goldfish GHRH-LPR	Fugu GHRH-LPR	Chicken GHRH-LPR	Zebrafish GHRHR	Goldfish GHRHR	Chicken GHRHR	Human GHRHR
Zebrafish GHRH-LPR	88	64	43	40	40	40	40
Goldfish GHRH-LPR		64	43	39	40	39	39
Fugu GHRH-LPR			44	39	40	40	40
Chicken GHRH-LPR				31	33	34	33
Zebrafish GHRHR					78	52	49
Goldfish GHRHR						54	51
Chicken GHRHR							60
Human GHRHR							

Amino acid identity was determined by using the ClustalW alignment program with BLOSUM62. The second form of zebrafish GHRH-LPR is not included, as it lacks 100 amino acids in the N-terminal domain compared with the receptors listed above.

physiological range. None of the peptides tested were able to stimulate VPAC1R in the IP pathway in the physiological range ($<10^{-6}$ M; Fig. 6C and D). PACAP and VIP are nearly equal in potency for the stimulation of the zebrafish VPAC1R ($EC_{50}=1.5-3\times10^{-7}$ M; Table 6) but the potency of the three peptides is greater for the PAC1R than for the VPAC1R.

PHIR had a different response pattern to PACAP and VIP compared with zebrafish VPAC1R or VPAC2R from other species. The PHIR had no significant response ($P>0.05$) to VIP, PACAP1, or PACAP2 in either the cAMP or IP pathways (Fig. 6E and F). In contrast to VPAC1R or VPAC2Rs, VIP does not stimulate the zebrafish PHIR, although the latter has high identity to VPAC2Rs of other species. However, the PHIR had a high potency in response to PHI in the cAMP assay with an EC_{50} value of 1.77×10^{-9} M (Fig. 6E). Hence, the PHIR is coupled to the G_s protein that activates the adenylyl cyclase pathway but is not coupled to $G_{q/11}$ protein that activates the IP pathway. Also, PHIR is distinct from the VPAC1R and VPAC2R in two ways: it is activated by PHI and is not activated by PACAP or VIP.

The physiological characteristics of the zebrafish receptors were compared with human VPAC2R using our zebrafish VIP and human VIP, two peptides with 82% identical amino acids. Zebrafish VIP was able to activate the human VPAC2R in both the cAMP (Fig. 6G) and IP (Fig. 6H) signaling pathways in a dose-dependent manner. It was striking in the cAMP pathway that human VIP and zebrafish VIP produced similar EC_{50} values with the human VPAC2R (7.6×10^{-9} M in response to human VIP and 5.4×10^{-9} M in response to zebrafish VIP; Table 6). Unexpectedly, zebrafish VIP also stimulated the human VPAC2R in the IP signaling pathway (Fig. 6H), as this VPAC2R primarily activates the cAMP path. VIP did not stimulate the zebrafish VPAC1R in the IP path as expected. Hence, zebrafish VIP is a potent stimulus for the human VPAC2R.

Also, the human VPAC2R was stimulated by zebrafish PHI. The EC_{50} was 7.4×10^{-9} M, which was not

significantly different from the EC_{50} for VIP suggesting a potent response (Fig. 6G). Part of the effect may be explained by the high sequence identity (78%) between zebrafish PHI and human PHM peptides, although zebrafish PHI was even more potent than human PHM ($EC_{50}=4.7\times10^{-8}$) with the human receptor. In short, zebrafish PHI and VIP are potent stimulators of the human VPAC2R.

Functional analysis of zebrafish GHRH-LPR and human GHRHR

To confirm the functional identity of zebrafish GHRH-LPR, we used three peptides: zebrafish GHRH-LP1, GHRH-LP2, and true GHRH. Each peptide was tested on the zebrafish GHRH-LPR expressed in COS7L cells. For comparison of the physiological profiles, human GHRHR was also expressed in COS7L cells. Both cAMP and IP production were measured after stimulation with different doses of peptides. Zebrafish GHRH-LPR responded to zebrafish GHRH-LP1 ($EC_{50}=3.3\times10^{-8}$ M) and to zebrafish GHRH (1.2×10^{-7} M) with no significant difference ($P>0.05$); the responses in the cAMP signaling pathway were dose dependent (Fig. 7) and similar to those in the chicken or goldfish system (Lee *et al.* 2007, Wang *et al.* 2007). However, there was no response of zebrafish GHRH-LP receptor to zebrafish GHRH-LP2 stimulation; likewise the goldfish receptor only responded to one of its two GHRH-like peptides (Lee *et al.* 2007). We found that human GHRHR had no response to either zebrafish GHRH-LP1 or zebrafish GHRH-LP2 but did respond to human GHRH as a positive control.

Tissue expression of zebrafish PAC1R, VPAC1R, PHIR, GHRH-LPR, GHRHR, and PHI/VIP mRNAs in adults

To investigate the expressions of zebrafish PHIR, GHRH-LPR, and PHI/VIP mRNAs in comparison with

zebrafish PAC1R, VPAC1R, and GHRHR mRNAs, RT-PCR was used for 11 tissues (Fig. 8). Zebrafish β -actin cDNA (1014 bp) was used as a control to verify the quality of the first-strand cDNA synthesized. The most widely distributed receptor is PHIR mRNA, which

is expressed strongly in all tissues. The VPAC1R-short mRNA (arrow in Fig. 8) was also widely expressed in adult zebrafish with stronger expression in the eye, brain, testis, gill, skin, and ovary; lower levels were detected in the heart, kidney, swimbladder, skin, and

Figure 3 (continued)

Figure 3 (A) Predicted structure of zebrafish PHI/VIP gene from the zebrafish genome data base in Vega blasted with our cDNA sequence. The gene size (3584 bp) is indicated on the right of the gene. Exons are numbered and are presented by closed boxes and a solid horizontal gray line indicates introns. The distance between the first five exons is indicated by numbers above the intron line. (B) cDNA and predicted amino acid sequences of zebrafish *vip* (PHI/VIP). The numbers on the right and left are the positions of the nucleotide sequences. The putative sequence of mature PHI and VIP are underlined and the putative PHM (C-terminal extension of PHI to the last residue (Met) before 'KR') is labeled with a black dot. The potential polyadenylation signals (AATAAA) are double underlined. (C) Amino acid sequence alignment of mature PHI and VIP peptides by ClustalW in vertebrates. Shaded areas represent identical amino acid residues. The sequences of goldfish A and B, chicken, mouse, and human are from Tse *et al.* (2002).

gut. The longer bands (arrow head in Fig. 8) are from PAC1R-hop mRNA, which has a hop cassette inserted in intracellular loop 3. PAC1R-short mRNA is more widely distributed than other forms of PAC1R mRNA in zebrafish (Fradinger *et al.* 2005). Also, PAC1R-short is the predominant isoform in goldfish (Kwok *et al.* 2006). VPAC1R mRNA was expressed in all 11 tissues with a lower level of expression in the gill, kidney, skin, and ovary. PHI/VIP mRNA was more strongly expressed in the eye, heart, brain, testis, gill, gut, and skin than in the swimbladder or ovary. GHRH-LPR and GHRHR mRNAs are distributed to only a few tissues. GHRH-LPR mRNA was only strongly expressed in eye, brain, testis, and kidney with very weak expression in swimbladder and heart. The GHRHR mRNA is mainly distributed in the brain, testis, and gut with very faint bands in the eye, kidney, and swimbladder. β -actin mRNA was expressed in a relatively similar manner in the 11 tissues.

Discussion

Proof that a novel zebrafish receptor is PHIR and not VPAC2R

Initially, we thought our newly isolated receptor of 2088 bp was a VPAC2R because it encoded a small section that was identical to a published fragment of VPAC2R (274 bp; Wang *et al.* 2003). Also, alignment of our deduced amino acid sequence with human and

chicken VPAC2R showed a sequence identity of 56 and 59% respectively. Signature motifs characteristic of human and chicken VPAC2R were present in the zebrafish sequence: Thirteen conserved cysteines including seven in the N-terminal extracellular domain, PDV, and RLAK binding motifs and other highly conserved amino acid residues for VIP binding (Fig. 1). Our phylogenetic analysis and chromosome linkage study also grouped this sequence with other VPAC2Rs.

In contrast to the mammalian VPAC2Rs, our receptor was not significantly stimulated by VIP even with a 10^{-5} M concentration analyzed in both cAMP and IP signaling pathways. Also, in the N-terminal extracellular domain that is fundamental for VIP binding (Laburthe *et al.* 2003), the zebrafish sequence is missing a potential glycosylation site, which is expressed in the human, chicken, and frog VPAC2R (Hoo *et al.* 2001). N-glycosylation sites are critical for ligand binding and correct delivery to plasma membrane of the human VPAC1R (Couvineau *et al.* 1995, 1996). However, the missing glycosylation site is not strong evidence for establishing the identity of our receptor, as the human VPAC1R functions correctly as long as two of the glycosylation sites are intact (Couvineau *et al.* 1996).

Finally, we discovered that our receptor has 80% amino acid sequence identity with goldfish PHIR; the goldfish sequence had been published (Tse *et al.* 2002) but not entered into the NCBI database. The goldfish

Figure 4 Maximum-likelihood phylogenetic analysis of zebrafish PHIR and GHRH-LPR amino acid sequences compared with other known related receptors. Only the seven transmembrane sequences were used in the alignment. Receptors identified in this report are highlighted in gray. Zebrafish glucagon receptor (GLUCR) was used as an outgroup. PHIR, peptide histamine isoleucine-receptor; GHRHR, growth hormone-releasing hormone receptor; GHRH-LPR, GHRH-like peptide receptor; PAC1R, PACAP1 receptor; VPAC1R and VPAC2R, VIP/PACAP shared receptors, types 1 and 2. *Receptors identified *in silico*, which have all seven TMDs but have a shortened N-terminal domain without an obvious start site.

PHIR is the only one characterized in vertebrates so far as we know. The sequence identity of our receptor with goldfish PHI receptor is higher than that for all reported VPAC2Rs (up to 65% in trout). Phylogenetic analysis also grouped the two fish PHIRs together. Moreover, in the N-terminal glycosylation site, goldfish has isoleucine in the same position as zebrafish (Fig. 1B; position 107, open circle) instead of asparagine needed for glycosylation. Based on functional studies (specific

response to PHI and no response to VIP or PACAP), sequence identity, and phylogenetic analysis, we conclude that this receptor is zebrafish PHIR. Our strongest evidence comes from functional analysis with PHI in which the receptor was activated in a range expected for the natural ligand ($EC_{50} = 1.77 \times 10^{-9}$ M). Further analysis of fish (e.g. fugu, medaka, and stickleback) VPAC2RA and VPAC2RB for PHIR characteristics is essential. First, to use fugu as an

Figure 5 Chromosomal location of *phir* for zebrafish and *VIPR2* (VPAC2R) for medaka, chicken, and human. Two linked genes (*ZMYND11* and *DIP2C*) in the same chromosomal region as the receptors are shown to be in corresponding positions for zebrafish, medaka, and chicken. However, in the human genome, *VIPR2* is on chromosome 7, whereas the 'linked' genes are on chromosome 10. *phir*, peptide histidine-isoleucine receptor; *vipr2*, VIP/PACAP shared receptor, type 2.

example, its VPAC2Rs only have two glycosylation sites each (Fig. 1B). Second, fugu VPAC2Rs share relatively high sequence identity with zebrafish PHIR: 55 and 62% of VPAC2RA and VPAC2RB respectively. Third, in NJ and ML phylogenetic trees, fugu VPAC2RA and VPAC2RB cluster more closely with zebrafish PHIR than with chicken and human VPAC2R. However, the sequence similarity may not represent functional equivalence as addressed by Cardoso *et al.* (2005). Whether fugu VPAC2RA and VPAC2RB are PHI receptors will be defined by functional assays.

One hypothesis is that the tetrapod VPAC2R and the teleost PHIR shared a common origin. Support for this hypothesis includes the close structural identity between the two types of receptors (Fig. 1), the chromosomal location and linked genes (Fig. 5), and the activation of the human VPAC2R in which zebrafish PHI ($EC_{50}=7.4 \times 10^{-9}$ M) is

more potent than human PHM ($EC_{50}=4.7 \times 10^{-8}$ M; Fig. 6E). Common ancestry is also suggested in that zebrafish PHI is a potent stimulator of both the zebrafish PHIR (1.8×10^{-9} M) and human VPAC2R (7.4×10^{-9} M; Fig. 6E and H). However, a phylogenetic map for peptide affinity changes cannot be prepared until a number of other so-called VPAC2Rs are tested with a species-specific PHI. This is especially true for the teleost fish where two forms of VPAC2Rs have been annotated in the genome; the duplicates are presumably due to a large scale or whole genome duplication. But the duplicate VPAC2Rs in fish has not yet been cloned and their start sites are ambiguous (Fig. 1). If the duplicates are functional, it is possible that one receptor is a PHI receptor and the other has a different function, possibly as a VPAC2R. Mapping of the receptor changes using affinity values to PHI, VIP, and PACAP should help to elucidate the proposed receptor status.

Figure 6 Accumulation of cAMP and inositol phosphate (IP) in COS7L cells transfected with different receptors and stimulated with graded concentrations of zebrafish (zf) and human peptides. (A) cAMP and (B) IP of zebrafish PAC1R; (C) cAMP and (D) IP of zebrafish VPAC1R; (E) cAMP and (F) IP of zebrafish PHIR; (G) cAMP and (H) IP of human VPAC2R. Values represent means $\pm$ S.E.M. from a minimum of three independent experiments each in triplicate. PACAP, pituitary adenylate cyclase-activating polypeptide; PHI, peptide histidine-isoleucine; PHM, peptide histidine-methionine; VIP, vasoactive intestinal polypeptide; PAC1R, PACAP receptor; VPAC1R, VIP/PACAP shared receptor, type 1; VPAC2R, VIP/PACAP shared receptor, type 2. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-08-0083>

Zebrafish PAC1R-short has unconventional response to VIP

It was generally accepted that PAC1R specifically binds PACAP with a very low affinity to VIP and that only VPAC1R and VPAC2R have similar affinity to both PACAP and VIP (Vaudry *et al.* 2000). This is true for PAC1R-short,

which is the basic form, and for the PAC1Rs that have added cassettes in the third intracellular loop. However, we show here that the PAC1R-short isoform in zebrafish has a significant dose-dependent response to PACAPs and VIP in both cAMP and IP assays. Although VIP is not as potent as the PACAP ($EC_{50} = 10^{-9}$ M) in activating the PAC1R, the EC_{50} value of zebrafish VIP is a physiological

Table 6 EC₅₀ values of receptors response to peptides

	zfPACAP1 isoform1	zfPACAP2 isoform2	zfVIP	zfPHI	zfGHRH-LP1	zfGHRH-LP2	zfGHRH
cAMP response EC ₅₀ (nM)							
zfPAC1R-short	1.23 ± 0.22	1.73 ± 0.54	34.4 ± 9.4	NR ^a			
zfVPAC1R	293 ± 90	154 ± 85	196 ± 130	984 ± 267			
zfPHIR	NR	NR	NR	1.77 ± 0.5			
hVPAC2R			5.4 ± 3.4	7.36 ± 3.25			
zfGHRH-LPR					32.6 ± 12.9	NR	115 ± 61.5
hGHRHR					NR	NR	
IP response EC ₅₀ (nM)							
zfPAC1R-short	46.2 ± 35.6	6.66 ± 2.58	668 ± 221				
zfVPAC1R	NR	NR	NR				
zfPHIR	NR	NR	NR	NR			
hVPAC2R			50.7 ± 14.8				
zfGHRH-LPR					NR	NR	
hGHRHR					NR	NR	
	humanVIP			humanPHM		humanGHRH	
cAMP response EC ₅₀ (nM)							
hVPAC2R		7.65 ± 3.24		46.6 ± 2.5			
hGHRHR						18.5 ± 14.6	

^aNR, no response at < 10⁻⁶ M peptide.

concentration (EC₅₀ = 4.7 × 10⁻⁸ M) and there was no significant difference ($P < 0.05$) between PAC1R and VPAC1R in response to VIP in the cAMP path. Support for the VIP action on the PAC1R comes from a recent paper on sea bream in which human VIP activated the sea bream PAC1R (EC₅₀ = 10⁻⁷ M), although less than the PACAPs (EC₅₀ = 10⁻⁹ M; Cardoso *et al.* 2007b). Relevant also is a report that there are at least 14 variants of the PAC1R in human neuroblastoma cells due to alternative splicing and some of these variants also respond to VIP stimulation in a dose-dependent manner in the cAMP and IP signaling pathways (Lutz *et al.* 2006). Likewise, human fetal brain has PAC1R variants that respond to VIP (Lutz *et al.* 2006). It is possible that certain variant PAC1Rs in both fish and human retained the function of responding to VIP. Moreover, human VPAC2R also responds well to zebrafish VIP with no significant difference ($P > 0.05$) compared with zebrafish PAC1R in both the cAMP and IP signaling pathways. The zebrafish VIP peptide shares 82% amino acid identity with human VIP.

Zebrafish GHRH-LPR responds to both zebrafish GHRH-LPR and GHRH

In the present study, we identified a new receptor in zebrafish, which we argue is GHRH-LPR because it has 88% sequence identity with goldfish GHRH-LPR but

only 40% identity with zebrafish and human GHRHR (Lee *et al.* 2007). Sequence alignment with goldfish and fugu GHRH-LPRs reveals closely related structures: 13 conserved cysteines, three N-glycosylation sites, and RLAK for the coupling to G_s and FQGGBVXXBYCFXN-XEXQ (B is a hydrophobic residue and X is any residue), characteristic of secretin family receptors. Also phylogenetic analysis grouped zebrafish and goldfish GHRH-LPRs along with other GHRH-LPRs with a bootstrap value of 100. The second form of zebrafish GHRH-LPR also clustered with the same group, as it has seven transmembrane domains but it lacks 100 amino acids at the N-terminus.

Functional assays revealed that the GHRH-LP receptors in both zebrafish and goldfish are most sensitive to one of their two GHRH-LPs but both also respond to their GHRH with about threefold less potency in zebrafish and 30-fold less in goldfish (Lee *et al.* 2007). The activation of the zebrafish GHRH-LPR was concentration dependent in the cAMP pathway and the potency of zebrafish GHRH-LP1 and zebrafish GHRH was not significantly different ($P > 0.05$). Similarly, Lee *et al.* (2007) found that the goldfish GHRH-LPR (PRPR) was stimulated by one of two goldfish GHRH-LPs, and by fish GHRH. The EC₅₀ values for fish GHRH tested in goldfish (1.8 × 10⁻⁷ M) and zebrafish (1.15 × 10⁻⁷ M) are nearly the same. Nonetheless, a more potent response occurs with GHRH-LP1; the EC₅₀

Figure 7 Functional assay using graded concentrations of zebrafish GHRH-LP1, GHRH-LP2, and GHRH on (A) cAMP and (B) IP accumulation in COS7L cells transfected with zebrafish growth hormone-releasing hormone-like peptide receptor (zebrafish GHRH-LPR); (C) cAMP and (D) IP accumulation in COS7L cells transfected with human GHRHR. Also, the latter was tested with human GHRH (cAMP). Values in cAMP assay represent means $\pm$ S.E.M. from three independent experiments each in triplicate. Values in IP assay represent means $\pm$ S.E.M. from at least two independent experiments each in triplicate.

values are 5.8×10^{-9} M for goldfish and 3.41×10^{-8} M for zebrafish. Moreover, neither zebrafish nor goldfish GHRH-LPR responded to its second form of GHRH-LP. We did not detect any response of human GHRHR to either form of zebrafish GHRH-LP. We conclude that zebrafish GHRH-LPR (PRP) has at least two specific ligands: zebrafish GHRH-LP1 and zebrafish GHRH in the cAMP path. However, zebrafish GHRH receptor only responds to fish GHRH, not to goldfish GHRH-LP stimulation (Lee *et al.* 2007).

The homologous receptor in humans (PRPR) has been lost from the human genome (Cardoso *et al.* 2007a, Lee *et al.* 2007). This implies that the function of GHRH-LP is distinct for fish and other non-mammals compared with mammals.

Cleavage of zebrafish prepro-PHI/VIP is predicted to produce two amidated peptides or extended forms without amidation

PHI and VIP are both transcribed from the same gene in vertebrates. PHI has an amino acid cleavage site of GKR, which implies that PHI is amidated at position 27. Another zebrafish peptide, PHM, is a C-terminal extension of PHI to a methionine at position 42 but is not amidated, as it is followed by only a KR cleavage site. VIP also has a GKR cleavage site after position 28. The zebrafish VIP C-terminus can be extended, which is similar to human VIP. Both PHI and VIP are amidated at the C-terminus but goldfish and chicken PHIs are not amidated because of the lack of a glycine residue (Talbot *et al.* 1995, Tse *et al.* 2002). Also, a VIP extension is absent in goldfish because VIP₂₈ is directly followed by a stop codon. The primary structure of zebrafish PHI has been conserved (78% amino acid identity) compared with the human PHM₂₇. In general, PHI/PHM activates all known mammalian VIP receptors (VPACR) with moderate to weak activation (Moriarty *et al.* 1984, Palle *et al.* 1989, Lelièvre *et al.* 1998). However, Lelièvre *et al.* (1998) suggested that PHI had its own specific receptor that preferentially binds PHI over VIP. In their study, PHI had a 100-fold more potent anti-proliferative action than VIP using radio-labeled PHI as a tracer for binding studies on neuroblastoma cells. The study also revealed that there were high-affinity receptors that selectively bound PHI over VIP, although neuroblastoma cells are known to express many alternatively spliced receptors with different binding profiles (Lutz *et al.* 2006). The existence of zebrafish and goldfish PHIR and the high conservation of PHI in fish and human suggest that both VIP and PHI play an important role in fish and possibly other vertebrates.

Figure 8 Tissue distribution of the zebrafish (zf) mRNA for PAC1R, VPAC1R, PHIR, PHI/VIP, GHRH-LPR, and GHRHR by RT-PCR. Zebrafish β -actin 1 is a positive control for each tissue examined. In the negative lane, no template was added. All PCR products spanned at least two exons. The arrow points to the PAC1R-short PCR product and the arrow head represents the PAC1R-hop product in different tissues. Abbreviations: PAC1R, PACAP1-specific receptor; VPAC1R, VIP/PACAP shared receptor 1; PHI, peptide histidine-isoleucine; VIP, vasoactive intestinal polypeptide; GHRH-LP, growth hormone-releasing hormone-like peptide.

Location of receptors indicates diverse target tissues for peptides

To compare the location of zebrafish PHIR and GHRH-LPR with other related peptide receptors, we performed an RT-PCR study. PHIR is one of the most widely distributed receptors and its mRNA highly expressed in all 11 tissues. The co-expression of PHIR with PHI in the eye, heart, brain, testis, gill, gut, swimbladder, skin, and ovary indicates that PHI may act in an autocrine/paracrine manner. Goldfish PHIR expression was quantified by real-time PCR (Tse *et al.* 2002). High expression levels were found in the brain, heart, testis, and gut, which are similar to zebrafish.

Zebrafish PAC1R-short mRNA is expressed widely except in the spleen. This result is similar to the observation of Fradinger *et al.* (2005), except we found additional weak expression in the heart and swimbladder. Goldfish PAC1R-short mRNA was not detected in the spleen either but has strong expression in the heart measured by real-time PCR (Kwok *et al.* 2006). The PAC1R-short isoform is one of the most widely distributed receptors tested here, along with PHIR in goldfish and zebrafish. In frog and mammal, the distribution was largely investigated by RT-PCR or *in situ*

hybridization in the brain and pituitary (Hashimoto *et al.* 1996, Shioda *et al.* 1997, Hu *et al.* 2000) where PAC1Rs are abundantly expressed.

By contrast, the distribution of zebrafish mRNA for GHRH-LPR and GHRHR are limited compared with other receptors studied here. This probably relates to tissue-specific functions. Zebrafish GHRH mRNA was only strongly expressed in brain, testis, and gut with weak signals in eye, kidney, swimbladder, skin, and ovary. GHRH-LPR mRNA was only detected in eye, brain, testis, and kidney with weak signals in the heart. But GHRH-LPR was expressed strongly in the eye compared with GHRHR. The distribution of zebrafish GHRH-LPR is comparable with a previous study in our laboratory (Fradinger *et al.* 2005).

Conclusions

The newly isolated zebrafish PHIR and GHRH-LPR contribute to a better understanding of the family B receptors from an evolutionary perspective. Characterization of these receptors helps to answer some of the questions posed in the Introduction. For question 1,

our data show that PHI does have a specific receptor although to date, it is known only for zebrafish and goldfish and not mammals. We suggest that PHIR and VPAC2R had a common ancestor, as they have high sequence conservation. Small changes in the common receptor, possibly after the fish–tetrapod split, may have transformed it to a PHIR or VPAC2R in evolution; one piece of evidence is that zebrafish VIP does not activate the zebrafish PHIR but does activate the human VPAC2R. To confirm the receptor change, many other PHI/VPAC2Rs in other species need to be tested with PHI, VIP, and PACAP to establish an affinity profile. In addition, the change in peptide structure during evolution may also be important, as zebrafish PHI not only activates its receptor but is more potent than human PHM when tested with the human VPAC2R in our experiments. Questions 2 and 3 about the lack of a specific VIP receptor suggest the possibility that the original PAC1R and VPAC1R responded to both PACAP and VIP, as in zebrafish. During evolution, PAC1R may have lost its sensitivity to VIP. However, humans do have a PAC1R variant that responds to VIP and PACAP; the variant was identified not only in neuroblastoma cells but in fetal brain (Lutz *et al.* 2006). PACAP and VIP may have shared a common origin, as the peptides have a high sequence identity of 68% for human PACAP versus hVIP and 78–81% for zebrafish PACAP1 or PACAP2 versus zebrafish VIP. In question 4 about the PRPR (GHRH-LPR), the ligand in all vertebrates regardless of its name is encoded by the PACAP gene. The structural similarity of PRP (and PHM) to GHRH was noted in an early paper describing the gene that encodes PRP/PACAP (Ohkubo *et al.* 1992). In human, the PRP sequence has a higher identity with GHRH₂₇ (52%) than with PACAP₂₇ (22%). In zebrafish, the same pattern occurs in which zebrafish GHRH-LP1 is closer in structure to zebrafish GHRH₂₇ (59%) than with zebrafish PACAP₂₇ (37%). The structural relationship of the upstream-encoded peptide in each of three genes (for PRP/PACAP, PHM/VIP and GHRH/C-terminal peptide) and also the downstream peptides of PACAP and VIP (see above) suggest the genes resulted from at least two duplications in evolution, which must have occurred before the fish–tetrapod split as teleosts have the same three genes as mammals. A substitution in the first amino acid of mammalian PRPs and the apparent loss of their receptors (Cardoso *et al.* 2007a, Lee *et al.* 2007) may have resulted in a hormone that no longer activates cAMP (Okazaki *et al.* 1992). Although the function of GHRH-LPs is not clear, the name should remain, as these peptides are structurally closer to GHRH than to PACAP. Hence, PRP is less a PACAP-related peptide than a PACAP gene-associated peptide. The answer to question 5 as to whether zebrafish is a good model for endocrine studies is shown in Table 1. These fish have a full complement of secretin superfamily

hormones except for secretin and its receptor, although the status of duplicate receptors is at an early state of study. The zebrafish superfamily has matching receptors for its known hormones including PHIR and GHRH-LPR (PRPR), unknown in humans to date.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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