Duplicated receptors for VIP and PACAP (VPAC₁R and PAC₁R) in a teleost fish, *Fugu rubripes*

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Abstract

Two principal groups of receptors orthologous with human PAC₁R and VPAC₁R and were identified and characterised at the genomic level in the teleost fish *Fugu rubripes*. An additional group orthologous with VPAC₂R was also identified and partially characterised. In *Fugu*, gene duplication of each of the PAC₁Rs, VPAC₁Rs and VPAC₂Rs appears to have occurred. The topology of the tree surrounding the *Fugu* duplications and other isolated piscine sequences indicates that the duplication events for these six genes clearly preceded the speciation event leading to the Cypriniformes and Tetraodontiformes and is probably teleost-specific. Overall, the combined pattern of gene expression for each pair of duplicated genes mirrored the expression in other vertebrates. However, within each pair of duplicates further specialisation had occurred, with each demonstrating differential tissue distribution profiles suggesting they that may be responsible for the divergent action of the ligands, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP). The *Fugu* VPAC₁R gene regions showed conserved synteny with human chromosome 3p21.3 and also *C. elegans* chromosome X, indicating that the putative ancestral human chromosome 3 region may be equivalent to chromosome X in *Caenorhabditis elegans*.

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Introduction

Vasoactive intestinal peptide (VIP) and the related peptide, pituitary adenylate cyclase-activating peptide (PACAP) belong to the glucagon hormone superfamily, which in humans includes secretin, growth hormone-releasing hormone (GHRH), glucagon, glucagon like-peptides 1 and 2 (GLP-1 and GLP-2), peptide histidine methionine (PHM) and glucose-dependent insulintropic polypeptide (GIP) (Sherwood *et al.* 2000). These peptides have been grouped into a superfamily because the precursor molecules share a strikingly similar structural organisation, similar amino acid sequences and in general the hormones are related in terms of distribution and function (Bell 1986, Sherwood *et al.* 2000). They function by binding to the N-terminal extracellular domain of a family of specific G-protein-coupled receptors (GPCRs). Members of this hormone gene family are

widespread in many phyla and have been identified in species as ancient as tunicates (McRory & Sherwood 1997). This has prompted the development of numerous models for this family's evolution (Sherwood *et al.* 2000) from a single ancestral molecule by the processes of gene and exon duplication.

The genomic structures and evolution of the receptors for these small ligands are not so well characterised. The VIP and PACAP receptors belong to a sub-set of G-protein-coupled receptors (the class II family) that includes the receptors for GHRH, corticotrophin releasing factor (CRF), calcitonin, secretin, parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) (Segre & Goldring 1993). The members of this family share amino acid sequence similarity in the seven transmembrane domains and a long N-terminal extracellular domain containing a number of cysteines that are important for ligand

binding (Segre & Goldring 1993, Laburthe *et al.* 1996). All the receptors mediate their action by activation of a G-protein that induces an intracellular signal cascade mediated by heteromeric GTP-binding proteins (Gilman 1987, Bockaert 1991, Ulrich *et al.* 1998). Ligand-binding studies have demonstrated, in humans, the existence of three VIP-interacting receptors: VPAC₁R, VPAC₂R and PAC₁R (the PACAP type 1 receptor). The VPAC receptors bind both PACAP and VIP ligands with similar affinities, whilst the PACAP type 1 receptor preferentially binds PACAP (Vaudry *et al.* 2000c). The two human VIP receptors, VPAC₁R and VPAC₂R, share less than 42% amino acid sequence similarity and have a widespread tissue distribution with different expression patterns for each of the receptors, as demonstrated by RT-PCR and *in situ* hybridisation studies. VPAC₁R is found mainly in the cerebral cortex, hippocampus, pineal gland, lungs and intestines and VPAC₂R, which is proposed to be the neuro-endocrine receptor for VIP, is found in the hypothalamus, pituitary, adrenal glands, pancreatic islets, testes and ovaries (Usdin *et al.* 1994, Vaudry *et al.* 2000a,b). In mammals, PAC₁R is expressed predominantly in the CNS, however the situation is more complex, with the production of alternative splice variants (hip-hop cassettes) which influence receptor selectivity for the PACAP38 and PACAP27 ligand isoforms (Spengler *et al.* 1993, Journot *et al.* 1994, Pantaloni *et al.* 1996, Chatterjee *et al.* 1997, Daniel *et al.* 1998, Dautzenberg *et al.* 1999). Isolation and functional characterisation of the PAC₁R in goldfish indicates that the PACAP ligand acts as a novel GH-releasing factor in this organism (Wong *et al.* 2000).

The isolation and characterisation of two VIP receptor subtypes (VPAC₁R and VPAC₂R) has been reported only in humans (Sreedharan *et al.* 1993, Gagnon *et al.* 1994), rat (Ishihara *et al.* 1992, Lutz *et al.* 1993) and mouse (Inagaki *et al.* 1994, Hashimoto *et al.* 1999) which raises interesting questions about when the gene duplication that gave rise to the two forms occurred. Relatively few receptors of this superfamily have been isolated from non-mammalian vertebrates (Peeters *et al.* 1999, Alexandre *et al.* 1999, Hu *et al.* 2000). In teleosts, only goldfish VPAC₁R and PAC₁R cDNAs have been isolated (Chow *et al.* 1997, Wong *et al.* 1998) and no genomic structures for these

receptors have been reported. In the present study the compact genome of the Japanese pufferfish (*Fugu rubripes*) was used to isolate and characterise the PAC₁, VPAC₁ and VPAC₂ receptor genes at the genomic level. The gene sequences obtained in the present study were also used to search for related genes in the invertebrate databases *Caenorhabditis elegans*, *Ciona intestinalis*, *Drosophila melanogaster* and *Saccharomyces cerevisiae* with the aim of identifying a putative ancestral VIP/PACAP receptor.

Materials and methods

Gene identification and isolation

During routine *Fugu* cosmid library sequence scanning (within the confines of the Fugu Landmark Mapping Project (Elgar *et al.* 1999)) a cosmid (C139D21) was identified as having a gene fragment with high sequence similarity to goldfish VPAC₁R (probability value in excess of $9e^{-19}$). PCR primers were designed for a conserved sequence motif of this family identified after a multiple sequence alignment of all the members of this family and the *Fugu* cosmid C139D21 partial sequence. A homologous PCR product was obtained with *Fugu* genomic DNA and used to screen the *Fugu* cosmid library (available from <http://www.hgmp.mrc.ac.uk>) at low stringency using 50 °C hybridization temperature in Church Gilbert buffer (1984), followed by a one minute wash at room temperature in Church Gilbert wash buffer. Twenty four further cosmids were isolated and characterised by sequence scanning as described in http://fugu.hgmp.mrc.ac.uk/Protocols/Biology/fugu_section2.html#2.1. The cosmids were sorted into different groups based on DNA sequence similarity, secondary screening of SacI restriction enzyme digest Southern blots and analysis of short-range linkage relationships. Gene content was determined by searching the individual sequence fragments using BLAST v2.0 (Altschul *et al.* 1997) against the SPTer (Bairoch *et al.* 1997) and Unigene databases (<http://www.ncbi.nlm.nih.gov/Unigene/Hs.home.html>).

Cosmids were identified for a single PAC₁R gene and for each of two distinct VPAC₁R-like and two VPAC₂R-like genes. Cosmids for each of the genes were subjected to full-depth sequencing. The cosmid containing PAC₁RA lacked the first exon, so this gene was completed using RACE PCR

(RACEEx4, 5'actctggacagttgacga3'). A further PAC₁R gene was identified from Fugu Consortium data (Aparicio *et al.* 2002) and mapped to two scaffolds: exons 2 and 3 mapped to scaffold S011651 and S004929 contained exon 6 to the 3' end. The first exon was identified using RACE PCR (PACAPP2 RACE2 5'accagatgggagcacttcggtcag) as a long series of Ns was present in scaffold S011651 5' to exon 2. Exons 4 and 5 were amplified using primers designed to exons 2 and 6 (PACAP2Ex3F, 5'ctgtggcatccagctcagat3'; PACAP2Ex6R, 5'tatgtatgaagcagtgctcgtt3'). Probing of a Southern blot, revealed the genomic distance of these two exons to cover approximately 1 kb, but the gene was completed by RT-PCR using *Fugu* brain cDNA. *Fugu* database mining revealed no further VIP or PACAP receptors.

For gene assembly, all sequences were transferred to a UNIX environment and quality clipped using a modified Pregap script (Bonfield and Staden 1996). Sequences were screened against sequencing and cloning (pBluescript and Lawrist4) vectors and matching regions were masked prior to further analysis. Edited sequences were then assembled using the Pint Assembly Programme (Weston P, HGMP, unpublished data, <http://menu.hgmp.mrc.ac.uk/cgi-bin/pint>). Contiguation and finishing of the gene was performed using PCR walks and sequencing of SacI cosmid sub-clones. All genes, unless stated in the results, were fully sequenced at the genomic level. Exons were determined by comparison with other vertebrate VPACR/PAC₁R sequences and searching for the GT/AG consensus sequences. Anomalous exon/intron boundaries were confirmed by RT-PCR.

The *Fugu* VPAC₁R, VPAC₂R and PAC₁R, genes have been submitted to the EMBL database with accession numbers AJ296144, AJ296143, AJ408877, AJ40887, AJ490804 and AJ494861 for VPAC₁RA, VPAC₁RB, VPAC₂RA, VPAC₂RB, PAC₁RA and PAC₁RB respectively.

***Fugu* VIPR/PACAPR gene analysis and database mining**

Analysis of the finished sequence was carried out using the HGMP Nix interface (G Williams, P Woollard & P Hingamp, unpublished data, <http://www.hgmp.mrc.ac.uk/NIX/>). Percentage of identities of the different genes was calculated with the Blosum 62 score table using the Genedoc

program (Multiple Sequence Alignment Editor and Shading Utility v2.6.001, (www.psc.edu/biomed/genedoc) and also using the needle programme from the EMBOSS suite of programmes (Rice *et al.* 2000) (<http://www.uk.emblnet.org/Software/EMBOSS/Apps/>) with a Gap opening penalty of 10.0 and a Gap extension penalty of 0.5. Comparative human gene map positions were determined using the Ensembl viewer (v1.0.0) (<http://www.ensembl.org/>) and gene names assigned using the HUGO nomenclature accessed via the Ensembl link to LocusLink (<http://www.ncbi.nlm.nih.gov/LocusLink/>).

Each of the *Fugu* VIP/PACAP receptor genes were used to search for similar genes in the human, zebrafish, *Tetraodon*, medaka, *C. elegans*, *D. melanogaster*, *C. intestinalis* and *S. cerevisiae* databases: (<http://www.ensembl.org/perl/blastview>; http://www.ensembl.org/Danio_rerio/; <http://www.genoscope.cns.fr/cgi-bin/recherche.cgi>; <http://shigen.lab.nig.ac.jp/medaka/genome/top.jsp>; http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml; <http://www.fruitfly.org/blast/>; <http://aluminium.jgi-psf.org/prod/bin/RunBlast.pl?db=ciona4> and <http://genome-www.stanford.edu/Saccharomyces/>). To establish if linkage groups had been maintained during the evolution of the VIP/PACAP receptor genes in *Fugu*, human and *C. elegans*, orthologous genes for the genes surrounding *Fugu* VPAC₁R, VPAC₂R and PAC₁R were searched and mapped in the human and *C. elegans* genomes using the <http://www.ncbi.org/LocusLink> and <http://www.wormbase.org/db/searches/basic#searchagain> web sites.

Phylogenetic analyses

Multiple sequence alignments were carried out using Clustal X (Thompson *et al.* 1997) and in the Genedoc program (Multiple Sequence Alignment Editor and Shading Utility v2.6.001, (www.psc.edu/biomed/genedoc)). Phylogenetic analysis was carried out from the Clustal X multiple alignment output using both the neighbour joining method (Saitou and Nei *et al.* 1987) via the PHYLO_WIN interface v1.2 (Galtier *et al.* 1996) and maximum likelihood method (Felsenstein 1985) via Phylyp v3.57c. Multiple alignment parameters for PHYLO_WIN: gap opening 15; gap extension 0.05; delay divergent sequences 40%; DNA transition weight 0.50, with 1000 bootstrap replicates. Maximum likelihood analysis was carried out using

the dnaml programme with default parameters and global rearrangements. The human GHRH clone (accession number, P43220) was used as the outgroup for the tree construction.

Tissue-specific expression

Tissue-specific expression of each of the genes was carried out by RT-PCR on eight tissues from different sexually immature juvenile fish (brain, gonads, gut, spleen, liver, kidney, heart and gills) generated by first strand synthesis of mRNA using the Promega Reverse Transcriptase kit according to the manufacturer's instructions. PCR was carried out on 1 µg cDNA using specific primer pairs designed for individual genes and spanning an intron as a control check for potential genomic contamination. Primers pairs for each gene: VPAC₁RA (V1AE4 – 5'gtaacctctccaagactggca3', V1AE5 – 5'tgcgatgaaa ccgtgggac3'); VPAC₁RB (V1BE2 – 5'ttcttctctgggaa tacgat3', V1BE8 – 5'tatggtgcccaaaatgggtgt3'); VPAC₂RA (V2AE3 – 5'agcgccgcggtcggagaggtca cg3', V2AE7 – 5'gccatgacaagtagtgaag3'); VPAC₂RB (V2BE4 – 5'atcttcccaacatcaccagc3', V2BE7 – 5'gcggttctcagagaagattac3'); PAC₁RA (P1AE2F – 5'gtggcgtcagagtgaatcac3', P1AE7R – 5'catcaccgctttgcacggc3') and PAC₁RB (P1BE3F – 5'ctgttggcatccagctcagat3', P1B6R – 5'tatgtatgaag-cagtgcgtt3'). PCR was performed with an initial denaturing step at 96 °C for 2 min, cycled 35 times (96 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min) with a final extension of 72 °C for 5 min. The VPAC₁RA PCR was performed at 67 °C with the same conditions described above. All PCR products of the correct size were sequenced to confirm their identity. To ensure that similar quantities of cDNA were used in all RT-PCR reactions, a further PCR with primers designed for ubiquitously expressed β-actin genes was also performed (data not shown).

Results

Screening of the *Fugu* genomic cosmid library with a probe generated by PCR to a conserved sequence motif of the family resulted in the identification of four different VIP receptors. They were named VPAC₁RA, VPAC₁RB, VPAC₂RA and VPAC₂RB, according to their sequence similarity with the other vertebrate orthologous genes. Due to

the difficulty in identifying the duplicated VIP receptor genes at the level of sequence scanning, the individual genes were characterised by their cosmid short-range linkage relationships (Table 1), sequence similarity and restriction digestion patterns. The A and B nomenclature was created to facilitate the distinction between the duplicated genes.

Fugu VPAC₁RA and VPAC₁RB genes

The *Fugu* VPAC₁RA and VPAC₁RB genes were completely sequenced at the genomic level and both code for a 419 amino acid protein, sharing 68% sequence identity. Comparison of the deduced amino acid sequence of VPAC₁RA and VPAC₁RB revealed they shared 51% and 49% sequence similarity with human VPAC₁R and 75% and 64% similarity to goldfish VPAC₁R, the only teleost in which the sequence is available. Structurally the *Fugu* VPAC₁R genes are composed of 12 exons and are 14.5 kb (VPAC₁RA) and 8.5 kb (VPAC₁RB) in length. Exon/intron positioning, splice site sequences and exon/intron boundary phases are identical between the two *Fugu* sequences. The two genes in *Fugu*, in common with the goldfish VPAC₁R gene (Chow *et al.* 1997), lack the first exon present in mammals that encodes the signal peptide sequence. To date, only two mammalian VPAC₁R genomic sequences from mouse (Hashimoto *et al.* 1999) and human (Sreedharan *et al.* 1993) have been characterised. These two genes are both composed of 13 exons and are 16 kb and 23 kb in length respectively. In comparative terms, the genes in *Fugu* are of a similar size (Fig. 1) and do not show the characteristic gene compaction of this organism (Brenner *et al.* 1993, Elgar *et al.* 1999). In general, with the exception of the absent first exon in both *Fugu* VPAC₁Rs, the gene organisation between human and *Fugu* is very similar, sharing the same splice sites, intron/exon boundary phases and location of the transmembrane domains. Sequence alignment of VPAC₁Rs from various species (Fig. 1B) indicate that they are very similar and signature motifs such as the RLAK motif between TM5 and TM6 which has been indicated for the functional coupling to Gsα (Okamoto *et al.* 1991) and the PDI motif typical of VIP binding receptors, were identified. Other motifs such as, the conserved R and L residues localised within TM2 which have

Table 1 VIP receptor genes in *Fugu*, humans (VPAC₁R and VPAC₂R) and *C. elegans* (AL031620) and their short-range linkage relationships. *Fugu* genes are listed according to their order in the *Fugu* genome. Human chromosome positions are indicated in brackets and the putative *C. elegans* homologous genes are characterised by their probability value obtained by sequence comparison and chromosome position.

Fugu VIPR and linked genes			Gene description	Human homologue gene	Putative <i>C. elegans</i> homologue genes
Hypothetical			Protein of unknown function	KIAA1173 (3p24.3-p22.1)	Z93395 (0.995) II
LOC131377			Kelch-related protein 1 (Sarcosin)	LOC131377 (3p21-32)	AF067946 (6.1e-32) V
NKTR			Natural killer tumor recognition protein	NKTR (3p23-p21)	U40938 (1.61e-30) X
SEC22C			Vesicle trafficking protein	SEC22C (3p24.3-p22.1)	Not found
VPAC ₁ RA			Vasoactive intestinal peptide receptor 1A	VPAC1R (3p22)	AL31620 (7.7e-27) X
CASQ			Calsequestrin muscle skeletal isoform precursor	CASQ (1q21)	Z69792 (1.5e-09) X
E2BE			Translation initiation factor EIF-2B epsilon subunit	E2BE (3q27.1-q24)	Z54284 (7.8e-12) II
VPAC ₂ RB			Vasoactive intestinal peptide receptor 1B	VPAC1R (3p22)	AL31620 (2.5e-27) X
LOC131377			Kelch-related protein 1 (Sarcosin)	LOC131377 (3p21-32)	AF067946 (6.1e-32) V
Hypothetical			Protein of unknown function	KIAA1173 (3p24.3-p22.1)	Z93395 (0.995) II
Hypothetical			Hypothetical protein of unknown function	KIAA0934 (10p15.3)	Not found
VPAC ₂ RA			Vasoactive intestinal peptide receptor 2A	VPAC2R (7q36.3)	AL31620 (1.5e-24) X
KIAA1585			Protein fragment of unknown function	KIAA1585 (3p12.3-q24)	Not found
TNSF10			Tumor necrosis factor ligand superfamily member 10	TNSF10 (3q26.31)	Not found
BS69			Adenovirus 5 E1-A binding protein	BS69 (10p14)	AF003389 (2.0e-10) II
Hypothetical			Hypothetical protein of unknown function	KIAA0934 (10p15.3)	Not found
KIAA1228			Protein Kinase conserved region 2-protein fragment	KIAA1228 (7q36.3)	U13019 (3.6e-29) III
VPAC ₂ RB			Vasoactive intestinal peptide receptor 2B	VPAC2R (7q36.3)	AL31620 (7.7e-30) X
PAC ₁ RA			Pituitary adenylate-cyclase activating polypeptide receptor 1	PAC ₁ R (7p14)	AL31620 (3.8e-30)
GHRHR			Growth Hormone Releasing Factor Receptor	GHRHR (7p14)	AL31620 (8.0e-23)
PAC ₁ RB			Pituitary adenylate-cyclase activating polypeptide receptor 1	PAC ₁ R (7p14)	AL31620 (3.8e-30)
GHRHR			Growth Hormone Releasing Factor Receptor	GHRHR (7p14)	AL31620 (8.0e-23)

B

Species	Sequence	Position
Human	MRPPSPPLPARWLCLAGALAWALGPAGGQAARLQ--EECDYVQMIEVQHKQCLEEEAQLNETTGCCKMWDNLTCPWPATPRGQVVVLACPLIFKLFSS--IQGRNVRSRCT	106
Rat	MRPPSPPHVRWLCLAGALACALRPAGSQAASPH--HECEYLQLEIQRQQCLEEEAQLNETTGCCKMWDNLTCPWPTTPRGQAVVLDCLPLFQLFAP--IHGYNISRST	106
Mouse	MRPPSLPPARWLCLAGALACALGPAGSRAASPH--OECEYLQMIKQKQCLEEEAQLNETTGCCKMWDNLTCPWPTTPRGQAVVLDCLPLFQLFSP--IHGYNISRST	106
Pig	MRPLSPPPAGWFCVLGVLGACVLGPGVSWAVGLQEECDYVQMIKQKQCLEEEAQLNETTGCCKMWDNLTCPWPATPRGQVVVLACPLIFKLFSS--TQGLNVRST	107
Chicken	-----MQSARLRVLLPGLGCLLSAASSTR-----PECSIMLEIEEERSQCLAEITEDNQTSGCRQWDNITCWPEAQVGAVVVKCPKPYRFLTT--FLG--NVSRNCT	95
Frog	-----MEFLPLLCLLTGLFSPILCVF-----EECSIMYQIELKHEECVNHEDYFNDTAVCKRTWDNITCWPSASIGEVVVLQCPGYFSMTTGTGVNG--NVSKNCT	94
Goldfish	-----MCDVNEIELARARCENKTAG--NVTSGCKGMWDIIACWPSAKVGEHVVPICPNYFRHFS--DHHEGNLSKSTCT	70
FuguA	-----MCDVAHDIELERERCENSVR--NVTAGCQGMWDIIACWPSAGVGEVVTITCPVYFSYFS--DQHKGNLSKSTCT	69
FuguB	-----MCDMKAELEBEKDTCEAQIE--NKTAGCTGMWDKITCWPSADVGEVVTVPCKPYLFFFSG--NTHLSNLSKSTCT	70
Human	DEGWTHLEPGPYPIACGLDDKAASLDEQQ--TMFYGSVKTGYTIGYGLSLATLLVATAILSLFRKLHCTRNYYIMHMLFISFILRAAAVFKDLALFDSG--ESDQCSEGSV	213
Rat	EEGWSQLEPGPYHIACGLNDRASSLDEQQTKFYNTVKTGYTIGYGLSLASLLVAMAILSLFRKLHCTRNYYIMHMLFMSFILRATAVFIKDMALFNNG--EIDHCSEASV	214
Mouse	EEGWSQLEPGPYHIACGLNDRASSMDEQQTEFYDAVKTYTIGYGLSLASLLVAMAILSLFRKLHCTRNYYIMHMLFMSFILRATAVFIKDMALFNNG--EIDHCSEASV	214
Pig	DEGWTHLEPGPYPIACGLDDKAASLDEQQ--TVFYNSVKTGYTIGYGLSLAALLVATAILSLFRKLHCTRNYYIMHMLFISFILRATAVFIKDLALFDSG--ESDHCSEGSV	214
Chicken	SQGWTDVYPA--YAVACGYDSNSTPGEEQ--TAFYCTVKTGYTIGYGLSLATLLVATAILSLFRKLHCTRNYYIMHMLFMSFILRAAAVFIKDLVLFESG--EPEHCPSVSV	200
Frog	SEGWSEMYPATYAAACGFSTNDTPTEQQ--TVFPGAIAKTGYTIGHSLSLISLTAAMIILCIPRKLHCTRNYYIMHMLFMSFILRAAAVFIKDLVLFESG--EIDHCSEGSV	200
Goldfish	ADGWTEMDPMEIAVYCGYNLNGTVDDDD--SFFRSVKIGYTGHSVSLSLITAVILCIMSRLHCTRNYYIMHMLFVSVFILKAIAVFKDAVLYDVQESDNCSTASV	175
FuguA	ADGWTEMDPMEIAVYCGYNLNGTVDDDD--NFWLQVQKIGYTVGYSVSLSLITAVILCIPRKLHCTRNYYIMHMLFVSVFILKAIAVFKDAVLYDVQESDNCSTASV	173
FuguB	EDGWSPIIS--IDYTEDCYDFNTTVGGDDRE--AGFFSAIKVGYTVGHSVSLSLITIGIILCLFRKLHCTRNYYIMHMLFVSVFILKAIAVFKDAVLYDVQESDNCSTASV	175
Human	GCKAAMVFFQYCVMANFFWLLVEGLYLYLTLAVSFFSERKYPFGWYILIGWGPVSTFMVWTTARIHPEDYGCWDTIN--SSLWIIKGPILTSILVNLFILFICIRILL	320
Rat	GCKAAMVFFQYCVMANFFWLLVEGLYLYLTLAVSFFSERKYPFGWYILIGWGPVSTFMVWTTARIHPEDYGCWDTIN--SSLWIIKGPILTSILVNLFILFICIRILL	322
Mouse	GCKAAMVFFQYCVMANFFWLLVEGLYLYLTLAVSFFSERKYPFGWYILIGWGPVSTFMVWTTARIHPEDYGCWDTIN--SSLWIIKGPILTSILVNLFILFICIRILL	322
Pig	GCKAAMVFFQYCVMANFFWLLVEGLYLYLTLAVSFFSERKYPFGWYILIGWGPVSTFMVWTTARIHPEDYGCWDTIN--SSLWIIKGPILTSILVNLFILFICIRILL	321
Chicken	GCKAAMVFFQYCVMANFFWLLVEGLYLYLTLAVSFFSERKYPFGWYILIGWGPVSTFMVWTTARIHPEDYGCWDTIN--SSLWIIKGPILTSILVNLFILFICIRILL	308
Frog	GCKAAMVFFQYCVMANFFWLLVEGLYLYLTLAVSFFSERKYPFGWYILIGWGPVSTFMVWTTARIHPEDYGCWDTIN--SSLWIIKGPILTSILVNLFILFICIRILL	307
Goldfish	GCKAAMVFFQYCVMANFFWLLVEGLYLYLTLAVSFFSERKYPFGWYILIGWGPVSTFMVWTTARIHPEDYGCWDTIN--SSLWIIKGPILTSILVNLFILFICIRILL	304
FuguA	GCKAAMVFFQYCVMANFFWLLVEGLYLYLTLAVSFFSERKYPFGWYILIGWGPVSTFMVWTTARIHPEDYGCWDTIN--SSLWIIKGPILTSILVNLFILFICIRILL	282
FuguB	GCKAAMVFFQYCVMANFFWLLVEGLYLYLTLAVSFFSERKYPFGWYILIGWGPVSTFMVWTTARIHPEDYGCWDTIN--SSLWIIKGPILTSILVNLFILFICIRILL	282
Human	QKLRPDPDIKNDSSPYSLAKSTLLLLIPLFGVHYIMFAFPDNFKAQVKMVFELVVGSGQGQFVVAIYLCFLNGEVCAELRRKWRWRWHLQGVLGWNPKYRHPSGGNGAT	429
Rat	QKLRPDPDIKNDSSPYSLAKSTLLLLIPLFGVHYIMFAFPDNFKAQVKMVFELVVGSGQGQFVVAIYLCFLNGEVCAELRRKWRWRWHLQGVLGWNPKYRHPSGGNGAT	431
Mouse	QKLRPDPDIKNDSSPYSLAKSTLLLLIPLFGVHYIMFAFPDNFKAQVKMVFELVVGSGQGQFVVAIYLCFLNGEVCAELRRKWRWRWHLQGVLGWNPKYRHPSGGNGAT	431
Pig	QKLRPDPDIKNDSSPYSLAKSTLLLLIPLFGVHYIMFAFPDNFKAQVKMVFELVVGSGQGQFVVAIYLCFLNGEVCAELRRKWRWRWHLQGVLGWNPKYRHPSGGNGAT	430
Chicken	QKLRPDPDIKNDSSPYSLAKSTLLLLIPLFGVHYIMFAFPDNFKAQVKMVFELVVGSGQGQFVVAIYLCFLNGEVCAELRRKWRWRWHLQGVLGWNPKYRHPSGGNGAT	417
Frog	QKLRPDPDIKNDSSPYSLAKSTLLLLIPLFGVHYIMFAFPDNFKAQVKMVFELVVGSGQGQFVVAIYLCFLNGEVCAELRRKWRWRWHLQGVLGWNPKYRHPSGGNGAT	416
Goldfish	QKLRPDPDIKNDSSPYSLAKSTLLLLIPLFGVHYIMFAFPDNFKAQVKMVFELVVGSGQGQFVVAIYLCFLNGEVCAELRRKWRWRWHLQGVLGWNPKYRHPSGGNGAT	393
FuguA	QKLRPDPDIKNDSSPYSLAKSTLLLLIPLFGVHYIMFAFPDNFKAQVKMVFELVVGSGQGQFVVAIYLCFLNGEVCAELRRKWRWRWHLQGVLGWNPKYRHPSGGNGAT	390
FuguB	QKLRPDPDIKNDSSPYSLAKSTLLLLIPLFGVHYIMFAFPDNFKAQVKMVFELVVGSGQGQFVVAIYLCFLNGEVCAELRRKWRWRWHLQGVLGWNPKYRHPSGGNGAT	391
Human	CSTQVSMLTRVSPGARRSS-SFQAEVSLV-----	457
Rat	CSTQVSMLTRVSPGARRSS-SFQAEVSLV-----	459
Mouse	CSTQVSMLTRVSPGARRSS-SFQAEVSLV-----	459
Pig	CSTQVSMLTRVSPGARRSS-SFQAEVSLV-----	458
Chicken	FSTQISMLTRVSPGARRSS-SFQAEVSLV-----	445
Frog	FSTQISMLTRVSPGARRSS-SFQAEVSLV-----	444
Goldfish	FSTQISMLTRVSPGARRSS-SFQAEVSLV-----	447
FuguA	FSTQISMLTRVSPGARRSS-SFQAEVSLV-----	417
FuguB	FSTQISMLTRVSPGARRSS-SFQAEVSLV-----	419

been suggested as being responsible for the receptor activation (Solano *et al.* 2001) and a highly conserved region at TM7 FQGBBVXXBYCFX NXEXQ which has been designated the consensus signature motif for the mammalian class II family members (Lok *et al.* 1994), where X and B represent any amino acid residue and any hydrophobic amino acid residue respectively, were also found in *Fugu*. In addition to these motifs, seven conserved cysteines at the 5' end and two conserved and three additional putative glycosylation sites were also identified in the *Fugu* genes (Fig. 1B).

Fugu VPAC₂RA and VPAC₂RB genes

Two different putative VPAC₂R genes were identified in *Fugu*, VPAC₂RA and VPAC₂RB. Genomic sequencing of each encompassed 7.4 kb and 12.2 kb respectively, to the 5' end of each cosmid. Neither of the cosmids containing the two VPAC₂R-like genes appeared to contain the 5' end of the genes, using homology searching and exon prediction software. Despite repeated attempts at RACE PCR, screening of the *Fugu* Consortium database (<http://fugu.hgmp.mcc.ac.uk>) and heterologous screening of a sea bream lambda cDNA library, it was not possible to identify the 5' terminal two exons of either VPAC₂R gene (12 exons were characterised). A sea bream clone for a VPAC₂R gene was isolated from the cDNA library, but this was truncated at the 5' end. Comparative analysis of the partial *Fugu* VPAC₂R genes with the VPAC₂R sequences from mouse, human, and rat revealed, as already observed for the VPAC₁Rs, the presence of seven conserved transmembrane domains (Fig. 2). The conserved motif characteristic of the VIP binding receptors,

P/A-D-V and the RLAK motif for the functional coupling to Gs α were also identified. As for *Fugu* VPAC₁R, the consensus signature identified for the mammalian class II family members at the TM7 FQGBBVXXBYCFXNXEVXQ was also identified (Fig. 2).

Fugu PAC₁RA and PAC₁RB genes

Two PAC₁R genes have been identified in *Fugu*. The first gene (PAC₁RA) was identified from heterologous probing of the *Fugu* cosmid filters and a further gene (PAC₁RB) was identified from the *Fugu* Consortium data (Aparicio *et al.* 2002). To date only one PAC₁RA receptor has been found in other vertebrates and previously only the genomic structure of rat and mouse have been characterised. The estimated sizes of each of the genes in *Fugu* was determined as 11.2 kb (PAC₁RA) and 5.7 kb (PAC₁RB), compared with 40 kb and 50+kb for rat and mouse respectively (Hashimoto *et al.* 1993, Aino *et al.* 1995), representing a varying compaction rate in *Fugu* (Fig. 3). Comparative analysis of the deduced amino acid sequence of *Fugu* PAC₁RA and PAC₁RB revealed that they are the most conserved of the three family duplications, sharing 74% sequence identity with each other and both share 69% sequence identity with human PAC₁R. However, such calculations are more complex with this receptor as the length of the PAC₁Rs varies between species from 465 amino acids in *Xenopus* to 523 amino acids in rat, due to the presence of the hip-hop cassettes which have only been sequenced in some species and were not identified in *Fugu*. The *Fugu* PAC₁Rs are both 444 amino acids in length and share the same genomic organisation. Comparative analysis of the *Fugu* PAC₁R genes with the PAC₁R sequences from other vertebrates

Figure 1 (A) Schematic representation of *Fugu* VPAC₁RA and VPAC₁RB genes and comparison with the orthologous human VPAC₁R. The gene sizes are indicated next to each gene on the right, exons are numbered and are represented by closed boxes and a solid black line indicates introns. Arrows between the *Fugu* VPAC₁R genes and the human VPAC₁R gene indicate equivalent exons. The largest intron in both *Fugu* genes is located between exons 2 and 3 and is 5 kb in VPAC₁RA and 2.5 kb in VPAC₁RB. Sizes are indicated next to each gene on the right, exons are numbered and are represented by closed boxes and the introns by a black line. (B) Clustal X alignment of the protein-coding region of *Fugu* VPAC₁R genes with other known VPAC₁Rs. The transmembrane domains are annotated above the sequence. Conserved cysteines are annotated with a circle and potential conserved N-glycosylation sites with a triangle. All motifs characteristic of the VPAC₁R genes are annotated with open boxes. Transmembrane domains (TM) and signal peptide sequences were identified by sequence comparison and according to GPCR database (www.cmbi.ni/7tm/) using the Swiss-Prot annotation. Accession numbers of the sequences are: mouse, P97751; rat, P97751; human, P32241; pig, Q28992; frog, Q9YHC6 and goldfish, Q90308. The chicken sequence is taken from Kansaku *et al.* 2001.

Figure 2 Transmembrane domains (TM) and signal peptide sequences were identified by sequence comparison and according to the GPCR database (www.cmbi.nl/7tm/) using the Swiss-Prot annotation. Clustal X alignment of the protein-coding region of *Fugu* VPAC₂R genes with other known VPAC₂Rs. The transmembrane domains are annotated above the sequence. Conserved cysteines are annotated with a circle and potential conserved N-glycosylation sites with a triangle. All motifs characteristic of the VPAC₂R genes are annotated with open boxes. Transmembrane domains (TM) and signal peptide sequences were identified by sequence comparison and according to the GPCR database (www.cmbi.nl/7tm/) using the Swiss-Prot annotation. Accession numbers of the sequences used are: mouse, P41588; rat, P35000; human, P41587.

revealed the presence of seven conserved transmembrane domains (Fig. 4). All potential conserved cysteines were present in the *Fugu* sequences and two conserved and two non-conserved N-glycosylation sites were also identified. The conserved motif characteristic of the PACAP binding receptors, PDM and the RLARS motif for the functional coupling to Gs α were also identified and a putative signal peptide sequence, albeit shorter than the sequence reported in mammals. As for the *Fugu* VIP receptors, the consensus signature identified for the mammalian class II family

members at the TM7 FQGBBVXXBYCFXNX EVXQ was also identified (Fig. 4).

Gene analysis and short-range linkage of *Fugu* VPAC/PAC₁ receptor genes

The gene environments linked to each of the *Fugu* VPACRs were different (Table 1) confirming the identification of four different VIP receptor genes in *Fugu*. Human VPAC₁R is located on chromosome region 3p22. *Fugu* VPAC₁RA was linked to four genes, all of which map in humans to

Figure 3 Comparison between rat PAC₁R and the two orthologous genes in *Fugu* (PAC₁RA; PAC₁RB). The gene sizes are indicated next to each gene on the right, exons are numbered and are represented by closed boxes and the introns by a black line.

chromosome 3p21·3. In humans, as in *Fugu*, these genes are neighbours with KIAA1173 directly adjacent to LOC131377, one gene is between LOC131377 and NKTR, one gene is present between NKTR and SEC22C, which is directly next to VPAC₁R. VPAC₁RB also showed conserved synteny to human chromosome 3. Both of the *Fugu* VIP receptor genes are linked to LOC131377 and KIAA1173, which clearly represents a regional duplication of all three genes. *Fugu* VPAC₂RA also showed linkage to genes which in human are present on chromosome 3, whilst a third linked gene (KIAA0934) maps to human chromosome 10p15·3. VPAC₂RB showed conserved linkage with a gene (KIAA1228) on human chromosome 7q, but interestingly was also linked to two genes (BS69 and KIAA0934), which map in humans to 10p. Once again, there is linkage of both VPAC₂R genes to an additional gene (KIAA0934), representing another small regional duplication in *Fugu*. The two PAC₁Rs identified from Consortium data were both present on short scaffolds containing only PAC₁R and GHRH, which both map to human region 7p14, and the linkage in *Fugu* clearly represents a duplication of both of these genes.

In order to search for additional members of this family of genes and to determine the conservation

of linked genes through evolution, the human, zebrafish, *Tetraodon*, medaka, *D. melanogaster*, *C. elegans*, *C. intestinalis* and *S. cerevisiae* databases were searched with the *Fugu* VIP/PACAP receptor coding sequences. The same sequences were also used to search for additional members in the *Fugu* genome. No additional members were found in the human and *Fugu* databases. In *Tetraodon*, duplicated genes were found for all the VPAC₁R, VPAC₂R and PAC₁R genes (in contigs 002569–2, 000637–2, 027090–1, 016424–1, 005290–1 and 004002–2 respectively, phylogenetic analysis not shown). It was possible to partially characterise these but the contigs lengths were too small to enable comprehensive short-range linkage analysis. Although the zebrafish genome data is currently available at 6x coverage, only two small fragments were putatively identified and designated VPAC₂A and PAC₁A after phylogenetic analysis (contigs ZC186F16 and ZC87 M5 respectively). The medaka genome data is available at 0·8x coverage and the contigs were too short to enable efficient gene identification. No putative members of the secretin/VIP/PACAP receptors were found in the *S. cerevisiae*, *C. intestinalis* and *D. melanogaster* databases.

In the *C. elegans* database three clones with the accession numbers U21309, Z11126 and AL031620 were found which shared high sequence

Figure 4 Clustal X alignment of the *Fugu* PAC₁R protein coding regions with other known PAC₁Rs. The transmembrane domains are annotated above the sequence. Conserved cysteines are annotated by a circle and potential conserved N-glycosylation sites are marked with a triangle. The alternatively spliced hop cassette is shown with a dotted box. Transmembrane domains (TM) and signal peptide sequences were identified by sequence comparison and according to the GPCR database (www.cmbi.nl/7tm/) using the Swiss-Prot annotation. Accession numbers of the sequences used are mouse, P70205; rat, P32215; human, P41586; bovine, D17290 and goldfish, AF048820. The chicken gene is taken from Peeters *et al.* 1999.

similarity to the *Fugu* VIP receptor sequences. These genes mapped within 6 Mb of each other. The *C. elegans* clone AL31620, located on chromosome X, was the only one with the highest identity for all of the *Fugu* VPAC/PAC₁ receptors (probability in excess of e-24) and a multiple sequence alignment (data not shown) of this clone with the other *Fugu* VPAC/PAC₁ receptors revealed that they shared high sequence similarity for the TM domains. This clone also shared conserved linkage for the calsequestrin gene with the *Fugu* VPAC₁RB. *Fugu* VPAC₁RA was also linked to a gene (NKTR), which in *C. elegans* mapped to the same chromosome (X) as the putative VPAC₁R clone and calsequestrin.

Phylogenetic analysis

Phylogenetic analysis was carried out on VIP/PACAP receptor genes using both the Neighbour Joining and Maximum Likelihood methods. Both methods produced the same relationships, of which the results from the Neighbour Joining method are shown in Fig. 5. The analysis clearly indicates that the 'extra' genes in *Fugu* clustered with the other VPAC₁R, VPAC₂R and PAC₁R genes and not with the closest family members (GHRH and secretin) and confirmed the initial identification made by sequence similarity and linkage. Of the genes considered, only a single PAC₁R and VPAC₁R have been previously identified and entered in the database from a teleost (goldfish). The topology of the tree surrounding the *Fugu* duplications and the goldfish sequences indicates that these duplication events preceded the speciation event leading to divergence of the Cypriniformes and Tetraodontiformes. Therefore it is expected that duplicate VPAC₁R, VPAC₂R and PAC₁R genes will be found in other teleosts. The relationship between the three duplications is difficult to determine with low bootstrap values and it is not possible to determine which of the receptors arose first.

Tissue distribution

RT-PCR was carried out using specific primers for each receptor in order to characterise the expression of each of the receptors in different *Fugu* tissues (data not shown). Although most of the receptors were expressed in gonads,

brain and gills, there was clearly a difference in the expression patterns between each of the duplicated genes.

Discussion

Fugu is the first organism in which duplication of PAC₁R, VPAC₁R and VPAC₂R has been identified. Comparison of the deduced amino acid sequences of the VIP/PACAP receptors from *Fugu* with other family members isolated from vertebrates revealed that they exhibited greatest sequence similarity to VIP/PACAP receptors and contained all the characteristic features and signature motifs of the class II GPCR family members. Structural comparison of *Fugu* receptors with the orthologous VPAC₁R and PAC₁R in humans shows that gene organisation has been highly conserved (as indeed was the case for the partial sections of the *Fugu* VPAC₂Rs). The main difference observed is that the first exon, which in mammalian VPAC₁R contains the signal peptide, is absent in both VPAC₁R receptors in *Fugu*. This situation is identical to that of goldfish, in which VPAC₁R was cloned as a cDNA and this exon is also absent, although the 5' UTR is present and the receptor is functional. Studies were carried out using goldfish VPAC₁R expressed in a mammalian cell line (Chow 1997) which showed that in goldfish the receptor stimulated the production of cAMP in the transfected cells, suggesting that the amino acids encoded by the first exon are not essential for receptor activity. It remains to be established if the fish VPAC₁R is integrated into the cell membrane and the existence of an alternative non-consensus signal peptide sequence in fish remains to be explored. Of the three duplicate receptors in *Fugu*, the PAC₁Rs are the most conserved in terms of sequence similarity and identity, they both encode a putative signal peptide, albeit shorter than that identified in other vertebrates so far. It was not possible to determine whether the *Fugu* VPAC₂R genes contained a signal peptide as the 5' end remains uncharacterised.

It is thought that all three receptors under study originated from a single ancestral gene which duplicated and subsequently diverged during the course of evolution (Ishihara *et al.* 1992, Lutz *et al.* 1993, Pisegna & Wank 1993, Inagaki *et al.*

Figure 5 Phylogenetic analysis of the *Fugu* VIP/PACAP receptor sequences with other known related receptors of the class II family. Names and abbreviations and accession numbers for all the sequences shown: rat VPAC₁, P97751; rat VPAC₂, P35000; rat PAC₁, P32215; rat sec, P23811; human V PAC₁, P32241; human VPAC₂, P41587; human PAC₁, P41586; human sec, P47872; human GHRH, P43220 (used as the outgroup); mouse VPAC₁, P97751; mouse VPAC₂, P41587; mouse PAC₁, P70205; frog VPAC₁, Q9YHC6; pig VPAC₁, Q28992; goldfish VPAC₁, Q90308; goldfish PAC₁, O73769; cattle PAC₁, Q29627; toad PAC₁, Q9PTK1; Fugu VPAC₁A, AJ296144; Fugu VPAC₁B, AJ296143; Fugu VPAC₂A, AJ408877; Fugu VPAC₂B, AJ408878; Fugu PAC₁A, AJ490804; Fugu PAC₁B, AJ494861; rabbit sec, O46502. The chicken sequences for VPAC₁ and PAC₁ were taken from Kansaku *et al.* 2001 and Peeters *et al.* 1999 respectively. Sec, secretin receptor.

1994). However, the topology of the initial branching between the PAC₁Rs, VPAC₁Rs and VPAC₂Rs is not clearly defined, with low bootstraps, and it is not possible to determine

which gene represents the more ancestral form of these receptors.

One theory about the evolution of the receptors is based on the chromosomal localisation of the

receptors in humans and rats. Both VPAC₂R and PAC₁R map to human chromosome 7 and rat chromosome 4, whereas VPAC₁R is located on human chromosome 3 and rat chromosome 8. A first duplication would produce the VPAC₁R gene and a common ancestor for VPAC₂R/PAC₁R. A second duplication event acting on the ancestral VPAC₂R/PAC₁R gene would produce the two separate genes (Cai *et al.* 1995, Sreedharan *et al.* 1995, Brabet *et al.* 1996, Mackay *et al.* 1996, Vaudry *et al.* 2000c).

Short-range linkage of the *Fugu* VIP receptor genes with human genes shows conserved synteny for some of the genes, especially for VPAC₁R-linked genes on human chromosome region 3p. This situation may be an indicator of a specific gene duplication event that occurred in this particular chromosome in the teleost lineage and so far the locus site for these receptors in fish has still to be mapped, so it is not known whether the *Fugu* VIP receptors linked to human chromosome 3 genes are very close on the same chromosome or on different chromosomes. VPAC₁RB shows conserved synteny for three genes LOC131377, E2BE and KIAA1173, the latter two of which in human are on opposite arms of chromosome 3, megabases apart. However, this is not surprising as studies in both zebrafish (Postlethwait *et al.* 2000) and *Fugu* (Bouchireb *et al.* 2001) have demonstrated that whilst fish may share large blocks of conserved synteny with humans, potentially extending over a whole human chromosome, the gene order can be radically altered by numerous inversion events. *Fugu* VPAC₂RA also shows similar linkage data to the *Fugu* VPAC₁R genes since most of the linked genes identified map to human chromosome 3. Whilst *Fugu* VPAC₂RB shares the same locus as the human VPAC₂R gene, as regards linkage to the KIAA1228 gene (7q36·3), there is stronger linkage to human chromosome 10p in the form of two genes linked to VPAC₂RB (BS69 and KIAA0934) and additionally VPAC₂RA is linked to a further duplicated KIAA0934. It is therefore clear that the duplication event that produced VPAC₂RA and VPAC₂RB also encompassed these KIAA0934 genes. These data support the idea that VPAC₁R and VPAC₂R arose from a gene duplication event of a common ancestor and suggest that in teleosts this duplication event occurred within the same chromosome followed by translocation of VPAC₂RB away from the putative ancestral

3p region. This was then followed by a further duplication to produce two separate genes VPAC₂R and PAC₁R, within an environment similar to human chromosome 7. This *Fugu* linkage data further supports the hypothesis of vertebrate VIP/PACAP receptor evolution based on human chromosomal locations described earlier (Cai *et al.* 1995, Sreedharan *et al.* 1995, Brabet *et al.* 1996, Mackay *et al.* 1996, Vaudry *et al.* 2000c).

Phylogenetic analysis suggests that VPAC₁RA and VPAC₁RB; VPAC₂RA and VPAC₂RB; and PAC₁RA and PAC₁RB in *Fugu* are duplicated forms of the VPAC₁R, VPAC₂R and PAC₁R in other vertebrates. Despite the relatively restricted number of species from which VIP receptors have been isolated, the topology of the phylogenetic tree suggests that duplication of the receptors in *Fugu* occurred prior to the branch that gave rise to mammals and, in line with current theories, is teleost-specific. In the goldfish and salmon, RT-PCR for VPAC₁R with degenerate primers amplified two partial cDNAs for VPAC₁R in both species. However, the authors only fully characterised one cDNA as the second fragment was assumed to arise because of the tetraploid nature of the genome in *Salmoniformes* and *Cypriniformes* (Chow *et al.* 1997). However, sequence comparison of the two distinct VPAC₁R cDNAs isolated from both salmon and goldfish with the *Fugu* VPAC₁Rs using phylogenetic methods clustered one of each of the goldfish and salmon cDNAs with *Fugu* VPAC₁RA and the others with *Fugu* VPAC₁RB (data not shown). Data-mining of other sequenced fish genomes further supports the timing of these teleost-specific duplication events, as orthologues of all the duplicated *Fugu* VIP receptors were identified in *Tetraodon* and a further two for VPAC₂A and PAC₁A were identified in zebrafish.

It is now a well-established fact that there are 'extra' genes in fish (Wittbrodt *et al.* 1998). However, the process by which these arose is still under debate. One proposal suggests that a further round of whole genome duplication has occurred in the fish lineage (Force *et al.* 1999, Taylor *et al.* 2001, 2003, Naruse *et al.* 2004, Vandepoele *et al.* 2004, Christoffels *et al.* 2004). Alternatively, these may have arisen due to a whole series of minor duplication events and not the single large-scale duplication originally proposed (Robinson-Rechavi *et al.* 2001a,b,c, Hughes *et al.* 2001). This data (together with the data mining and anecdotal

Figure 6 Comparative maps showing conserved linkage between *Fugu*, human and *C. elegans*. The chromosome positions of the genes in human and *C. elegans* are given in Mb and annotated next to the gene name. KIAA1173, LOC131377, NKTR, SEC22C and VPAC₁R in human all map to a gene cluster on 3p21-32 which contains only two other genes.

evidence in other fish species) clearly shows that there has been a minimum of several large chromosomal segmental duplications in teleosts involving the VIP receptors, producing further support for the hypothesis of an extra whole genome duplication having taken place in the teleost ancestor. Identification and characterisation of duplicated genes, particularly gene family members, provides important data towards the more large-scale, class-level (Teleostei) phylogenetic analyses cited above. Analysis of these large data sets, whilst necessary for a complete understanding of genome evolution, can contain weaknesses, as the history of specific gene families may not be completely understood or investigated, rendering gene loss and local gene duplications difficult to take into consideration. Analysis of a particular gene family (similar to that presented here) can identify these small-scale events. Whilst the issue of an extra whole genome duplication event in fish remains a contentious issue, the preponderance of polyploid genomes in fish certainly complicates

genome analysis and a resolution to this issue. It also raises a further issue, as to why these events occurred and the benefits for the organisms involved. It is generally acknowledged that gene duplication has played a significant role in the metazoan radiation. Further duplications in the fish could have fuelled their incredible speciation, as they comprise over half of all vertebrate species and unlike mammals their genomes (and the ploidy levels) are not constrained by a rigid sex chromosome system. The procession of duplicated genes with differential expression patterns may also, to a certain extent, obviate the need for alternative splicing.

The four VPAC and 2 PAC₁ receptors identified in *Fugu* were used to search for receptor homologues in the *C. elegans* genome (Fig. 6). A single gene localised on chromosome X that shared a high level of sequence similarity with all the *Fugu* VPAC₁Rs was identified and assumed to be an ancestral form of VIP/PACAP receptor (AL31620, probability value higher than e^{-24} using BLASTX). It was

considered that short-range gene linkage analysis might help to substantiate the phylogenetic model of how the VIP receptors evolved from the ancestral form in *C. elegans*. *Fugu* VPAC₁RA is linked to the gene calsequestrin, which is also present in *C. elegans* (Z69792) on the same chromosome as the putative ancestral VPAC₁R. Additionally, VPAC₁RA shows linkage to another gene (NKTR) which maps in *C. elegans* (U40938) to chromosome X. Taken as a whole, this data potentially indicates that the ancestral VPAC₁R gene and immediate gene environment in *C. elegans* was on chromosome X (Fig. 6) and that this may be equivalent to the putative ancestral human chromosome 3 region.

The widespread tissue distribution of the two VIP receptors in mammals may explain why VIP has such a wide spectrum of activity. In mammals it is an important regulator of the digestive tract (Ulrich *et al.* 1998), it is also involved in the immune (Pozo *et al.* 2000), reproductive (Usdin *et al.* 1994), circulatory (Fahrenkrug 1993) and neuro-endocrine systems (Vijayan *et al.* 1979, Biancani *et al.* 1985, Sherwood *et al.* 2000).

In fish, far fewer studies of VIP function exist but it has been proposed to be of importance in osmoregulation (Mainoya and Bern 1984, Chow 1997). The tissue distribution of the receptor in *Fugu* should provide clues about the potential activities of VIP in fish. In fact, *Fugu* VIP receptors have a tissue distribution reminiscent of that observed for VPAC₁R and VPAC₂R in mammals (Hashimoto *et al.* 1993, Usdin *et al.* 1994). The *Fugu* VPAC receptors are all present in gonads with varied expression profiles in the other different tissues (VPAC₁RA is additionally present in spleen, brain, kidney and gills; VPAC₁RB in brain; VPAC₂RA in heart and gut and VPAC₂RB present in all tissues tested except gonads). Both VPAC₁R and VPAC₂R were identified in mammal heart and gut (Ishihara *et al.* 1992, Gagnon *et al.* 1994, Usdin *et al.* 1994) but in *Fugu* only VPAC₂R was present. RT-PCR in *Fugu*, using primers specific for each receptor form, demonstrated that there is some overlap in tissue distribution of the duplicated VPAC₁R genes in brain and gonads and of the duplicated VPAC₂R genes in gonads, heart and gut. The only other study in fish is on goldfish VPAC₁R (Chow 1997) but it is unclear if the primers used in the study hybridised to other forms of the receptor, making it difficult to directly compare *Fugu* and goldfish VPAC₁R tissue

distribution. Surprisingly although VPAC₁R has been detected in the gut of human, rat and goldfish (Sreedharan *et al.* 1993, Usdin *et al.* 1994, Chow 1997) it was absent in *Fugu*. This may merely reflect the position of the sample of *Fugu* gut collected, as VIP receptors have a differential distribution in this tissue (Sayadi *et al.* 1988, Korman *et al.* 1989). It should be noted that further overlap of tissue expression may occur between these six receptors, as the RT-PCRs were only carried out on single tissues from juvenile fish and developmental differences may also occur. In view of the fact that *Fugu* tissue availability is limited, expression and functional studies of these receptors is being further characterised in a more amenable teleost, *Sparus aurata* (sea bream).

The PACAP ligand is remarkably well conserved in evolution between protochordates and mammals indicating that it is involved in important biological functions (McRory & Sherwood 1997). It shows a wide tissue distribution in mammals, notably in the brain and in peripheral organs such as the pancreas, gonads and respiratory and urogenital tracts (Vaudry *et al.* 2000c). Similarly, the PACAP type 1 receptor shows a broad spectrum of tissue expression, however it is predominantly expressed in the CNS (D'Agata *et al.* 1996, Basille *et al.* 2000) where it is thought to act not only as a hypophysiotropic hormone, but also as a neurotransmitter and/or neuromodulator (Hashimoto *et al.* 1993, Ogi *et al.* 1993, Peeters *et al.* 1999). The correlation between ligand and receptor distribution is more difficult to make in the case of PACAP compared with the VIPs, as the PACAP ligand can also interact with the VIP receptors. The distribution in *Fugu* is interesting as the two receptors show quite different distribution patterns, although overall the expression patterns of the two receptors combined in *Fugu* mirror the tissue distribution in mammals. PAC₁RB shows a universal tissue distribution on all tissues tested except for the gonads, which is very similar to the situation found in mammals. Whereas PAC₁RA is much more restricted in that it only shows expression in the brain, gonads and gills, with predominant expression in the former two tissues. Clearly there has been a redefinition of expression patterns between these two receptors, with PAC₁RA developing the more specialised role. The only other study in fish is on goldfish PAC₁R (Wong *et al.* 1998). RT experiments revealed a

generalised low level of expression throughout all 11 tissues tested, with predominant expression in the heart, brain and pituitary. However, the identities of the RT products were only confirmed by hybridisation and therefore they could have potentially cross-hybridised with both receptor forms, making it difficult to directly compare the *Fugu* and goldfish PAC₁R tissue distribution. The phylogenetic analysis of the receptors indicates that the goldfish sequence is the orthologue of the *Fugu* PAC₁RA and therefore, based on the results presented here, expression of the goldfish receptor would be expected to have a restricted tissue distribution. Further work will be required on a wider range of teleosts to determine if there is species-specific tissue distribution of the PAC₁Rs, which reflects physiological differences between fish.

The reason why VPAC₁R, VPAC₂R and PAC₁R genes have persisted in duplicate in *Fugu* and have overlapping expression patterns is still unknown. Under the classical model of evolution, duplicate genes survive by altering function, either by one becoming a pseudogene, by amino acid alterations or complementary degenerate mutation of the promotor regions to produce alternate and maybe very different functions (sub-functionalisation) (Force *et al.* 1999, Lynch & Conery 2000). Promotor analysis of these genes in *Fugu* has not yet been carried out, due to lack of 5' genomic data for some of the genes, but considering the tissue distribution, whilst each gene may perform a similar overall function, they may have alternate distinct tissue-specific functions specific to the fish lineage.

Gene evolution is a complex issue, which in fish is further complicated by the plasticity of the genome and capacity to accommodate 'extra' genes. However, comparison of genomic regions in humans, fish and more ancient organisms can help to reconstruct ancestral genomic regions. Although fish diverged from the tetrapod lineage approximately 450 Ma, it would be wrong to assume that their development has been static. Genes in fish, which are also present in human, have been adapted throughout that 450 Ma span to accommodate their different lifestyles in aquatic and land environments. Interestingly, despite extensive database searching, heterologous probing and degenerate PCR experiments, it has not been possible to identify either a secretin receptor or ligand in *Fugu*

and therefore we propose that the duplication that gave rise to secretin and its receptor occurred after the divergence of fish from the tetrapod lineage 450 Ma. The exact nature of the function of the four VIP and two PACAP receptor genes in *Fugu* described here, and their relationship to the mammalian orthologues remains to be determined, but no doubt will produce interesting results about the adaptation to the aquatic environment and osmoregulation.

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