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Identification of a non-mammalian leptin-like gene: Characterization and expression in the tiger salamander (*Ambystoma tigrinum*)

Timothy Boswell *, Ian C. Dunn, Peter W. Wilson, Nerine Joseph, David W. Burt, Peter J. Sharp

Division of Genetics and Genomics, Roslin Institute (Edinburgh), Roslin, Midlothian EH25 9PS, UK

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Abstract

Leptin is well established as a multifunctional cytokine in mammals. However, little is known about the evolution of the leptin gene in other vertebrates. A recently published set of ESTs from the tiger salamander (*Ambystoma tigrinum*) contains a sequence sharing 56% nucleotide sequence identity with the human leptin cDNA. To confirm that the EST is naturally expressed in the salamander, a 409 bp cDNA was amplified by RT-PCR of salamander testis and stomach mRNAs. The coding sequence of the cDNA is predicted to encode 169 amino acids, and the mature peptide to consist of 146 residues, as in mammals. Although the overall amino acid identity with mammalian leptins is only 29%, the salamander and mammalian peptides share common structural features. An intron was identified between coding exons providing evidence that the sequence is present in the salamander genome. Phylogenetic analysis showed a rate of molecular divergence consistent with the accepted view of vertebrate evolution. The pattern of tissue expression of the leptin-like cDNA differed between metamorphosed adult individuals of different sizes suggesting possible developmental regulation. Expression was most prominent in the skin and testis, but was also detected in tissues in which leptin mRNA is present in mammals, including the fat body, stomach, and muscle. The characterization of a salamander leptin-like gene provides a basis for understanding how the structure and functions of leptin have altered during the evolution of tetrapod vertebrates.

Keywords: Leptin; *Ambystoma tigrinum*; Salamander; Amphibian; Molecular evolution; Gene expression

1. Introduction

It is now over 10 years since the discovery of the adipose tissue hormone leptin in the mouse and human was reported (Zhang et al., 1994). It was initially thought that the primary function of leptin was to signal body fat content. However, subsequent research has indicated that it is a multifunctional cytokine of much broader significance. In the context of energy balance regulation, leptin is of primary importance in co-ordinating the neuroendocrine response to starvation (Ahima and Flier, 2000). Additionally, it is involved in the hormonal regulation of a diversity of physiological functions (Ahima and Flier, 2000; Wauters et al., 2000), including reproduction (Caprio et al., 2001), cardiovascular function (Rahmouni and Haynes, 2004), and bone development (Gimble and Nuttall, 2004). Its cytokine actions are reflected in its role as a regulator of inflammatory processes and immune response (Otero et al., 2005).

In the first report describing the cloning of the leptin gene in the mouse and human, a Southern blot was presented that showed hybridization of a mouse leptin cDNA probe to genomic DNA from the chicken and the eel, as

* Corresponding author. Present address: Division of Biology, King George VI Building, University of Newcastle, Newcastle-upon-Tyne, NE1 7RU, UK. Fax: +44 0 191 222 5228.
E-mail address: timothy.boswell@ncl.ac.uk (T. Boswell).

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well as to representatives of several Orders of eutherian mammals (Zhang et al., 1994). This suggested that the leptin gene is conserved among vertebrate Classes. Owing to its potential physiological importance, leptin has attracted considerable interest from researchers studying non-mammalian vertebrates. However, in 10 years since its discovery, there has been little evidence that a gene resembling mammalian leptin is present in the genome of any non-mammalian vertebrate. Two laboratories in the late 1990s reported the cloning of a leptin cDNA in the chicken that shared 95 and 97% sequence identity with mouse leptin at the nucleotide and amino acid level, respectively (Ashwell et al., 1999; Taouis et al., 1998). However, several independent laboratories have since been unable to amplify the reported sequence by RT-PCR (Amills et al., 2003; Friedman-Einat et al., 1999; Pitel et al., 2000). Doubts about its validity were reinforced by the results of comparative Southern blotting experiments, and by molecular evolutionary analysis (Dunn et al., 2001; Friedman-Einat et al., 1999). Moreover, no genomic sequence has been produced to confirm the existence of the cDNA, and no sequences resembling it are present in the chickEST database (http://www.chick.umist.ac. uk), or in the recently completed chicken genome sequence (http://www.ncbi.nlm.nih.gov/genome/guide/chicken). There is, however, evidence that a leptin-like signaling system is present in birds because a receptor has been cloned in the chicken that shows conservation of the key motifs and predicted exon boundaries found in the long isoform of the mammalian leptin receptor (Horev et al., 2000; Ohkubo et al., 2000). The endogenous ligand for this receptor has yet to be identified.

The lack of leptin-like genomic sequences in non-mammalian species has necessarily restricted investigation into leptin's molecular and functional evolution to studies of the cloned mammalian genes (Chmurzynska et al., 2003; Doyon et al., 2001; Gaucher et al., 2003; Hope et al., 2000). However, the application of genomics to non-mammalian vertebrates is allowing previously uncharacterized leptin-like genes to be identified. Very recently, a leptin-like gene was isolated in the pufferfish (*Takifugu rubripes*) (Kurokawa et al., 2005). This was achieved by identification of the region in the pufferfish genome that exhibited conservation of synteny with markers surrounding the human leptin gene. Use of the *Takifugu* leptin sequence to search fish genome databases enabled similar sequences to be identified in the river pufferfish (*Tetraodon nigroviridis*) and the Japanese medaka (*Oryzias latipes*) (Kurokawa et al., 2005). A leptin-like sequence was also identified in the Atlantic salmon (*Salmo salar*) EST database (Kurokawa et al., 2005). Genomic resources are also being applied to non-mammalian tetrapod vertebrates. This is exemplified by two recent amphibian EST sequencing projects that together have generated almost 60,000 cDNA sequences from the tiger salamander (*Ambystoma tigrinum*) and the axolotl (*A. mexicanum*) (Habermann et al., 2004; Putta et al., 2004). Some evidence is available for the existence of a leptin signaling system in amphibians in that leptin-like immunoreactivity has been observed in the stomach of the African clawed frog (*Xenopus laevis*) and in the gastrointestinal tract and neural tube of a newt (*Triturus cristatus carnifex*) (Buono and Putti, 2004; Muruzábal et al., 2002). Furthermore leptin-like gene sequences have now been identified in the *Xenopus tropicalis* genome database (Kurokawa et al., 2005) and a similar *Xenopus laevis* sequence has been deposited in GenBank (Accession No. AY884210). This led us to search the ESTs recently generated in ambystomatid salamanders for leptin-like sequences. We identified one EST in the tiger salamander and an EST contig in the axolotl that shared 98% nucleotide sequence identity between the two species, and 56% identity between the salamander and human leptin cDNA sequence. The aim of the present study was therefore to characterize and confirm the expression in the tiger salamander of a non-mammalian leptin-like gene.

2. Materials and methods

2.1. Animals and tissues

Three captive-bred tiger salamanders were obtained from a United Kingdom dealer having been maintained under artificial lighting and temperature in moist aquaria. To facilitate comparison with mammalian studies, metamorphosed, terrestrial adult, animals were chosen for investigation. On arrival, the salamanders were weighed and measured before being killed in accordance with the United Kingdom Home Office Code of Practice by immersion in 0.1% tricaine methane-sulfonate (ethyl 3-aminobenzoate methane-sulfonate) anaesthetic (Sigma, Poole, Dorset, UK). The three salamanders used in the study comprised a male weighing 22.5 g with a snout to tail-tip length of 17 cm and two females with respective body masses and lengths of 22.4 g/16.5 cm and 14.5 g/13 cm. None of the animals had reached full sexual maturity. Food was present in the gastrointestinal tract of all individuals at the time of dissection indicating that the animals were in a fed state.

2.2. Nucleic acid extraction

Total RNA was prepared from dissected tissue using RNA-Bee (AMS Biotechnology, Abingdon, Oxfordshire, UK) according to the manufacturer's instructions. Individual tissue blocks were homogenized in Lysing Matrix D using a FastPrep Instrument (Qbiogene, Bingham, Nottingham, UK). Poly(A)$^+$ RNA was isolated from total RNA using an Oligotex kit (Qiagen, Crawley, West Sussex, UK). Genomic DNA for PCR studies was isolated from liver using a GenomicPrep Cells and Tissue DNA isolation kit (Amersham Biosciences, Chalfont St. Giles, Bucks, UK).

2.3. PCR

For analysis of tissue distribution of leptin gene expression, reverse transcription was performed on 3 μg total RNA using a first-strand cDNA synthesis kit (Amersham) with *Not*I-d$(T)_{18}$ as a primer. PCR primers were designed from a Tiger salamander leptin-like EST deposited in GenBank (Accession No. CN054256). The primers used were: forward primer 5′-ATCCCAAC CTTCCACTGTC (positions 90–108 of Accession No. CN054256) and reverse primer 5′-ACCTATCCAACG CAACTTTC (positions 479–498), yielding a reaction product of 409 bp. To confirm the effectiveness of reverse transcription, PCR was also carried out to amplify a cDNA fragment of cyclophilin A. Primers were designed from an axolotl (*Ambystoma mexicanum*) EST (Accession No. BI818006). The forward primer was 5′-C TTCACAAACCACAATGGAAC (positions 212–232 of BI818006) and the reverse primer was 5′-ACAGAT GAAAAACTGGGAGC (positions 340–359), yielding a reaction product of 148 bp. PCR for both leptin and cyclophilin A cDNAs was performed using FastStart *Taq* polymerase (Roche Diagnostics, Lewes, East Sussex, UK) in PCR buffer containing 2 mM $MgCl_2$ on a Hybaid MBS system thermocycler block with an annealing temperature of 60 °C and denaturing and extension steps of 94 °C and 72 °C, respectively. Times used were 15 s denaturation, 30 s annealing, and 30 s extension, with an extension time for the final cycle of 5 min. PCR was carried out for 30 cycles for cyclophilin A and 35 cycles for leptin. PCR was also used to amplify a fragment of the salamander leptin gene using the Roche Expand Long Template PCR kit and 50 ng genomic DNA as template. The primers used were as described above for leptin cDNA amplification. The denaturation, annealing, and extension temperatures and times were 94 °C, 10 s; 63 °C, 30 s; and 68 °C, 5 min; with a final extension step of 7 min. PCR was carried out for 25 cycles. PCR amplification products were resolved by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The sequences of the leptin and cyclophilin A cDNA and leptin genomic DNA fragments amplified by PCR were confirmed by ligating the respective amplification products into the *Eco*RV site of pBluescript II SK+ (Stratagene, Cambridge, UK), transforming into *Eschericha coli* XL1-Blue MRF (Stratagene), and sequencing by a dideoxy termination method on a LI-COR 4200 sequencer (LI-COR Biosciences, Cambridge, UK). Nucleotide sequence assembly was performed using Wisconsin Package Version 10.0 (Genetics Computer Group, Madison, Wisconsin, USA). For leptin cDNA, sequence was obtained in both directions from six clones amplified from testis, and six clones amplified from the stomach of a different animal. For cyclophilin A, sequence was amplified from kidney, skeletal muscle, and liver, each of these tissues being taken from a different animal. Three clones were sequenced for kidney and skeletal muscle, and two clones for liver, and each clone was sequenced in both directions. For genomic DNA, two clones were isolated from different individuals, and these were each sequenced three times in both directions. Leptin sequence analysis is presented in Section 3 and the cDNA and genomic sequences obtained have been deposited in GenBank with Accession Nos. DQ064637, DQ064639, and DQ064640. The 148 bp cyclophilin A cDNA fragment shared 99% nucleotide sequence identity with the axolotl cyclophilin A EST (Accession No. BI818006) and 89% identity with *Xenopus* cyclophilin A (Accession No. AY646834, Masse et al., 2004). The sequence has been deposited in GenBank with the Accession No. DQ064638.

2.4. Northern analysis

Poly$(A)^+$ RNA was extracted using a commercial kit (Oligotex, Qiagen) from total RNA (isolated as described above) prepared from testis. Five micrograms was electrophoresed through a 1.4% agarose gel containing 1× Mops (10× Mops = 0.2 M 3-(*N*-morpholino) propanesulfonic acid, 80 mM sodium acetate, 10 mM disodium EDTA, pH 7.0) and 0.67 M formaldehyde. The gel and 1× Mops running buffer each contained 0.01 μg/ml ethidium bromide. RNA size markers were used to determine transcript size and the gel was photographed under UV light before blotting. The RNA was transferred to a nylon membrane (Hybond XL, Amersham) using downward denaturing capillary transfer for 2 h with 5× SSC, 10 mM sodium hydroxide as transfer buffer. The membrane was baked for 2 h at 80 °C. Hybridization was carried out using Ultrahyb solution (Ambion, Huntingdon, Cambridgeshire, UK) according to the manufacturer's instructions before being prehybridized in Ultrahyb for 30 min at 68 °C in a hybridization oven. A salamander leptin riboprobe was synthesized by in vitro transcription from the T3 promoter of a plasmid consisting of the 409 bp leptin cDNA fragment, amplified by RT-PCR as described above, ligated into the *Eco*RV site of pBluescript II SK+ (Stratagene) and linearized by digestion with *Hin*dIII. In vitro transcription was performed using T3 polymerase (Roche) and [^{32}P]UTP (800 Ci/mmol, Amersham) was incorporated as label to a specific activity of 8×10^{17} Bq/mole. The riboprobe was added to 10 ml Ultrahyb at a final concentration of 1×10^6 cpm/ml and hybridization was performed overnight at 68 °C. The membrane was then rinsed in 2× SSC, 0.1% SDS (2 × 5 min), followed by 0.01× SSC, 0.1% SDS (2 × 15 min) at 68 °C. The washed membrane was exposed overnight to a phosphor-imaging screen and digital images were captured using a Molecular Imager FX and Quantity One software (Bio-Rad, Hemel Hempstead, Hertfordshire, UK).

2.5. Tertiary structural analysis

The tertiary structure of salamander leptin was predicted by comparative modelling. The SWISS-MODEL server (http://swissmodel.expasy.org//SWISS-MODEL.html) was probed in the Alignment Interface with a ClustalW alignment (http://www.ebi.ac.uk/clustalw) of the following mammalian protein sequences: mouse (P41160); rat (P50596); human (P41159); cow (P50595); pig (Q28603); dog (O02720); fat-tailed dunnart (Q9XSW9) together with the salamander sequence. The target sequence of salamander leptin was modelled against the selected template human sequence (1AX8.pdb). Images of the predicted structures were analyzed using the SwissPdb Viewer (Guex and Peitsch, 1997).

2.6. Molecular phylogeny

A phylogenetic tree was constructed on the basis of non-synonymous substitutions for leptin in human (NM_000230), sheep (U84247), fat-tailed dunnart (AF067726), mouse (U18812), dog (AB020986), pig (AF052681), *Xenopus laevis* (AY884210), salmon (BI468126), and cow (U43943) sequences. These were aligned using Pileup (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, Wisconsin, USA), checked to ensure they were in the same translation frame, and the rate of non-synonymous substitutions was calculated using the Diverge program (Wisconsin Package Version 10.0). The trees were created using Growtree (Wisconsin Package Version 10.0) with the unweighted pair group method using arithmetic averages.

3. Results

3.1. Cloning and structural analysis of tiger salamander leptin

Primers were designed to amplify a 409 bp fragment of salamander leptin cDNA and the resulting sequence information was compared to the salamander EST clone deposited in GenBank (Accession No. CN054256). The cDNA fragment isolated by PCR (Accession No. DQ064637) was 99.5% identical to the EST sequence, differing by a G to A substitution at position 409, and an A to G substitution at position 476. The former substitution resulted in a conservative amino acid coding change from arginine to glutamine. The consensus salamander leptin cDNA spans 823 bp and includes the entire predicted coding sequence of a peptide containing 169 amino acid residues (Fig. 1). The primers used for amplification of the cDNA generated a 6 kb fragment from PCR of genomic DNA template (Fig. 2). This fragment was not sequenced completely, but partial reads were obtained from both ends. The first comprised 105 bp of the 3′-end of a putative exon that shared 100% identity to the cDNA sequence, followed by 405 bp of putative intronic sequence (Accession No. DQ064639). The second sequence consisted of 164 bp of putative intronic

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AATTCCCGGGATGTTTAAGGCCTCTCCAGAAACATCTGTGGAAAATGCGCTCCTCTGGCTTATCTCTCCTTGGATTCCTC    80
                                                M  R  S  S  G  L  S  L  L  G  F  L    12

TGGATGTGGatcccaaccttccactgtcGGCCAATCATGGTGGACCAACTCAGAATGGATGCCAAAAACCTCACCAGAAC   160
 W  M  W  I  P  T  F  H  C  R  P  I  M  V  D  Q  L  R  M  D  A  K  N  L  T  R  T    39
                                    Exon boundary
CATCATGGCCAGACTCCAGGAGCATCCAAGCCAG┆TTCCTTCTCCCGATGAACCTGAAGGTGAGCGGCTTAGACTTCATTC   240
  I  M  A  R  L  Q  E  H  P  S  Q ┆F  L  L  P  M  N  L  K  V  S  G  L  D  F  I     65

CAGGAGAGCAGTCCCTGGAGAGTTTGGACTCAGTAGACGAAACCCTAGAGATCTTCCATGCCATCCTCTCCAGCCTACAC   320
P  G  E  Q  S  L  E  S  L  D  S  V  D  E  T  L  E  I  F  H  A  I  L  S  S  L  H    92

ATGGACAACATGGAACAGATCCTCAGCGACATTGAGAACCTCCGGCGCCTTCTCCATGCCTTAAGTTCTCTGCTTGGCTG   400
 M  D  N  M  E  Q  I  L  S  D  I  E  N  L  R  R  L  L  H  A  L  S  S  L  L  G  C   119

CAATGCCCAGAAGAGCGTGCATCCAGACACTCTGGGGAACCTGACAGAAGAGTATGCCAAGTCTCCATTCACAACGGAga   480
  N  A  Q  K  S  V  H  P  D  T  L  G  N  L  T  E  E  Y  A  K  S  P  F  T  T  E     145

aagttgcgttggataggtTTCAGAAGAACCTTCACAGCATTGTCAAACATTTGGAGCATACCTTGAGCTGCTGAAGGGAA   560
K  V  A  L  D  R  F  Q  K  N  L  H  S  I  V  K  H  L  E  H  T  L  S  C  *          169

CAAAAACAGACTCTGGGATATTGAACCCCACCATGAACATGGTTGTGTCCAACTCCACAATGCTATTGTAAGAAAAAGCA   640

CTCTAAACCACAGACTCCCAAATTGGGAGGGCTTGTGGATCCTTCGGCCCACAAACATCTTCGAGGGAAAAAGTGAACCG   720

AAGCTCTGGCAATGTACCCCCATACTAACAAAGGGGTCAAAGGCTCAGGAGGTTACACAGCACTCATGACAGCAATGGGT   800

TTCCAGTTGACCTGGGAAAACTC                                                            823
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Fig. 1. The nucleotide and predicted amino acid sequence of tiger salamander leptin cDNA. The sequence is derived from an EST (Accession No. CN054256) and, between nucleotide sequence positions 90–498, from sequencing of RT-PCR products amplified from testis and stomach (Accession No. DQ064637). The primers used to amplify the 90–498 fragment are shown in bold lower-case. The existence of a boundary between exons was confirmed by sequencing of genomic DNA and is denoted by a dashed line between nucleotide positions 194–195. The termination codon is marked with an asterisk.

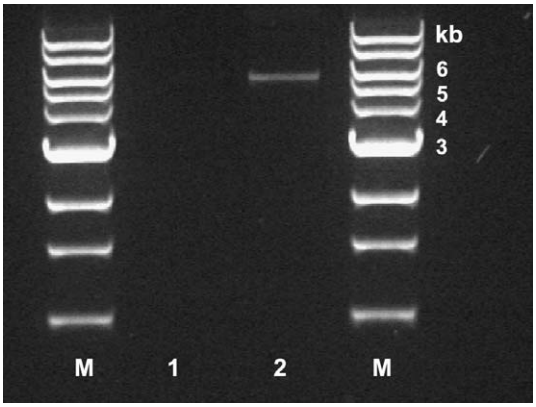

Fig. 2. Amplification of salamander genomic DNA using the primers shown in Fig. 1. A 6 kb amplification product is generated (lane 2). M designates size marker; lane 1 represents no template control; lane 2 shows amplification from 50 ng salamander genomic DNA template.

sequence contiguous with 304 bp of the putative 5′-end of an exon that shared complete identity with the cDNA sequence (Accession No. DQ064640). Splice donor and acceptor sites were predicted at the proposed intron–exon boundary with confidence scores >0.9 by splice site prediction programs (SSPNN, http://www.fruitfly.org/seq_tools/splice.html, and NetGene2, http://www.cbs.dtu.dk/services/NetGene2/). This exon–intron boundary lies within the coding sequence (Fig. 1) and corresponds to the position of the boundary between exon 2 and intron 2 in the mammalian leptin gene. From the size of the amplified genomic DNA fragment, the intron isolated in the salamander is approximately 5.5 kb in length. When compared to mammalian leptin cDNA sequences, the 507 bp salamander leptin cDNA nucleotide coding sequence shows 57% identity over 492 bp with the pig, and 56% identity over 479 bp with the human. When the predicted amino acid sequence of salamander leptin is compared with the leptin sequences of representatives of different mammalian Orders, 48 residues are shared in all species analyzed, representing an overall sequence identity of 29% between salamander and mammalian leptins (Fig. 3). The closest similarity to a mammal is with a marsupial, the fat-tailed dunnart (*Sminthopsis crassicaudata*). This sequence shares 61 residues with the salamander, representing a similarity of 36%. The salamander sequence shares 101 conserved residues with a *Xenopus laevis* leptin-like sequence (Accession No. AY884210), a sequence similarity of 60% (Fig. 3). Sequence identity between the salamander and *Xenopus* is 66% at the nucleotide level over the coding sequence. When the *Xenopus* sequence is included in the multiple protein sequence analysis, 40 residues are shared among tetrapod leptins, representing 24% sequence similarity (Fig. 3). The leptin sequence in all tetrapod species includes conserved cysteine residues in positions 119 and 169 that form a disulfide bond. While the residues conserved between amphibians and mammals are generally spread quite evenly, two prominent conserved regions lie between salamander residues 61 and 66 and between residues 102 and 113 (Fig. 3). The least conserved parts of the sequence include the pre-

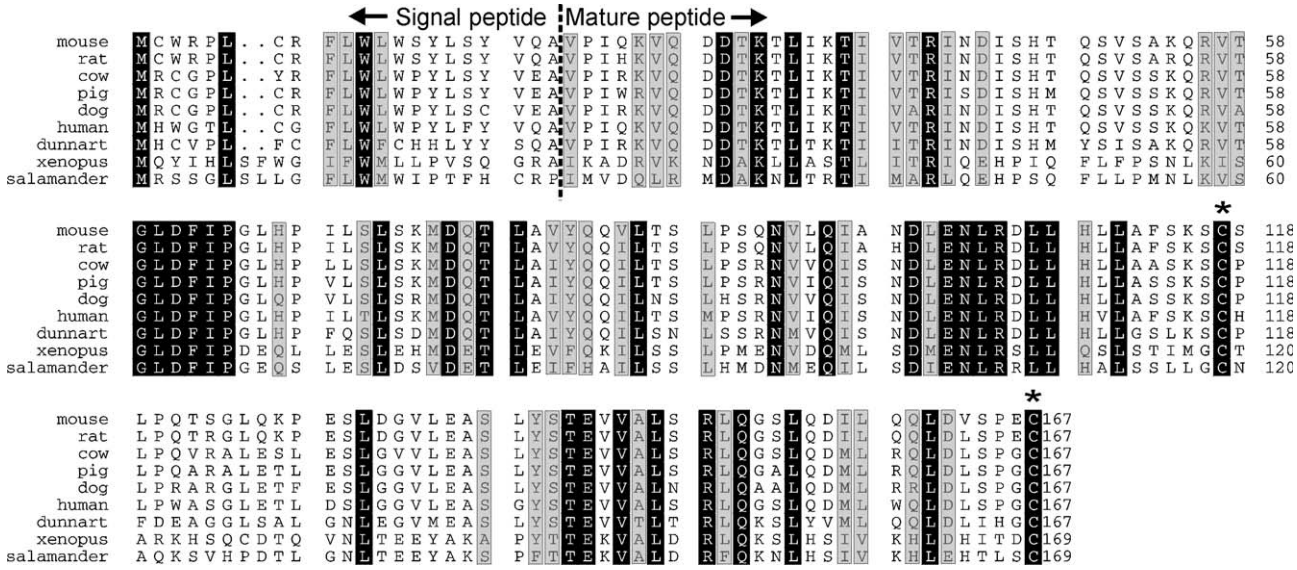

Fig. 3. Multiple amino acid sequence alignment of leptin from the mouse (Accession No. P41160), rat (P50596), cow (P50595), pig (Q29406), dog (O02720), human (P41159), fat-tailed dunnart (*Sminthopsis crassicaudata*, a marsupial) (Q9XSW9), *Xenopus laevis* (AY884210), and tiger salamander. Alignment was performed by Pileup (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, Wisconsin, USA). Amino acids common to all species are highlighted in black and conservative substitutions in grey. Note that all mammalian sequences comprise 167 amino acids while salamander and *Xenopus* leptins comprise 169. Conserved cysteine residues (positions 119 and 169 of the salamander sequence) that constitute a disulfide bond are indicated by overlying asterisks. The mature peptide begins at position 24 of the salamander sequence as indicated.

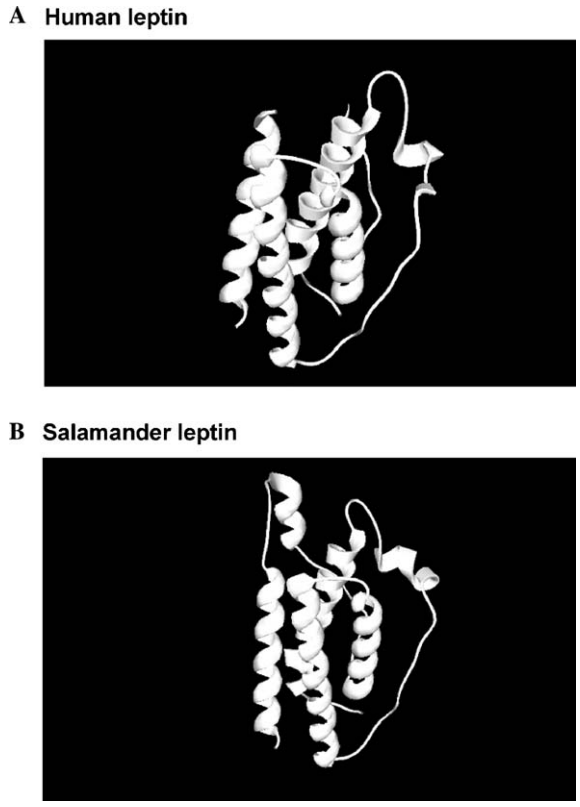

Fig. 4. The predicted tertiary structure of human (A) and salamander (B) leptin proteins. Structures were predicted using SWISS-MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html) in Alignment Interface with human leptin (1AX8.pdb) and a ClustalW alignment of mammalian leptins as templates.

dicted signal peptide (residues 1–23) and a region lying between residues 114 and 141. Modelling of the predicted salamander leptin protein indicates a tertiary structure of a bundle of four main helices (Fig. 4). The salamander model deviated from the human structure with a root mean square deviation of 0.25 Å, indicating that the predicted salamander protein has a tertiary structure comparable to mammalian leptins.

3.2. Molecular phylogeny

Construction of a phylogenetic tree based on the rate of non-synonymous substitutions in fish, amphibian and mammalian leptins demonstrates that the evolutionary relationship between leptin molecules is consistent with the consensus view of vertebrate evolution (Fig. 5) and that the salamander leptin sequence identified is unlikely to be an artifact.

3.3. Northern analysis

Northern hybridization of 5 μg poly(A)$^+$ RNA from salamander testis with the salamander leptin cDNA probe yielded a single band of RNA with a molecular size of 1.9 kb (Fig. 6).

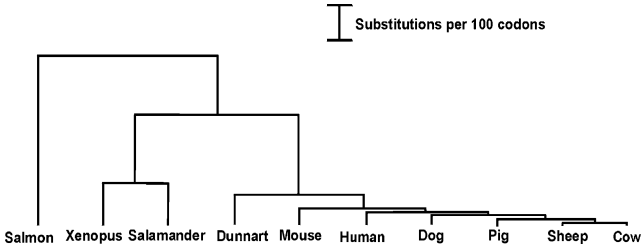

Fig. 5. Phylogenetic tree constructed using non-synonymous substitutions for salamander and mammalian leptins. Trees were created by Growtree (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, Wisconsin, USA) with the unweighted pair group method using arithmetic averages. Sequences used were human (NM_000230), sheep (U84247), fat-tailed dunnart (AF067726), mouse (U18812), dog (AB020986), pig (AF052681), *Xenopus laevis* (AY884210), salmon (BI468126), and cow (U43943).

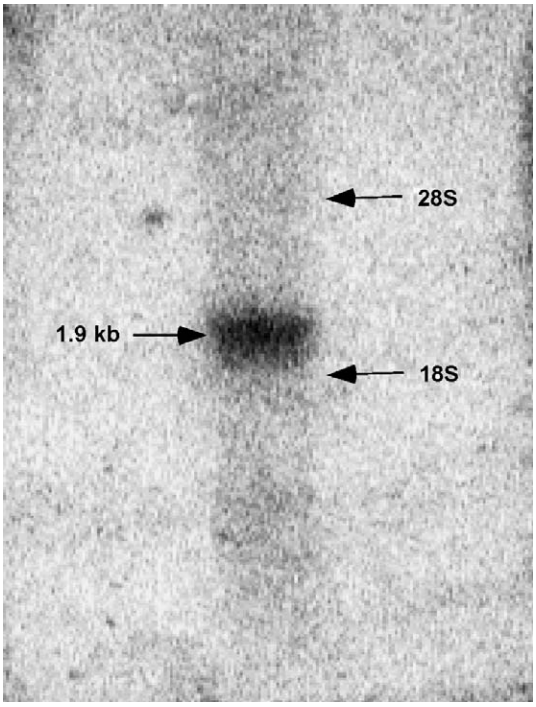

Fig. 6. Northern hybridization of leptin poly(A)$^+$ RNA prepared from salamander testis. A 5 μg aliquot of poly(A)$^+$ RNA was electrophoresed through a 1.4% agarose formaldehyde gel, transferred to a nylon filter, and hybridized with a ^{32}P-labeled antisense salamander leptin riboprobe. The hybridization signal was captured digitally after overnight exposure to a phosphor-imaging screen. The transcript detected is 1.9 kb in size. Position of the 18S and 28S ribosomal RNA bands is shown for reference.

3.4. Tissue distribution of leptin expression

The distribution of salamander leptin expression was studied by RT-PCR on mRNA isolated from a variety of tissues in three individual animals (Fig. 7). Leptin expression differed between the individuals studied. The most widespread expression was observed in the smallest animal, a female (Fig. 7A). The strongest intensity of the

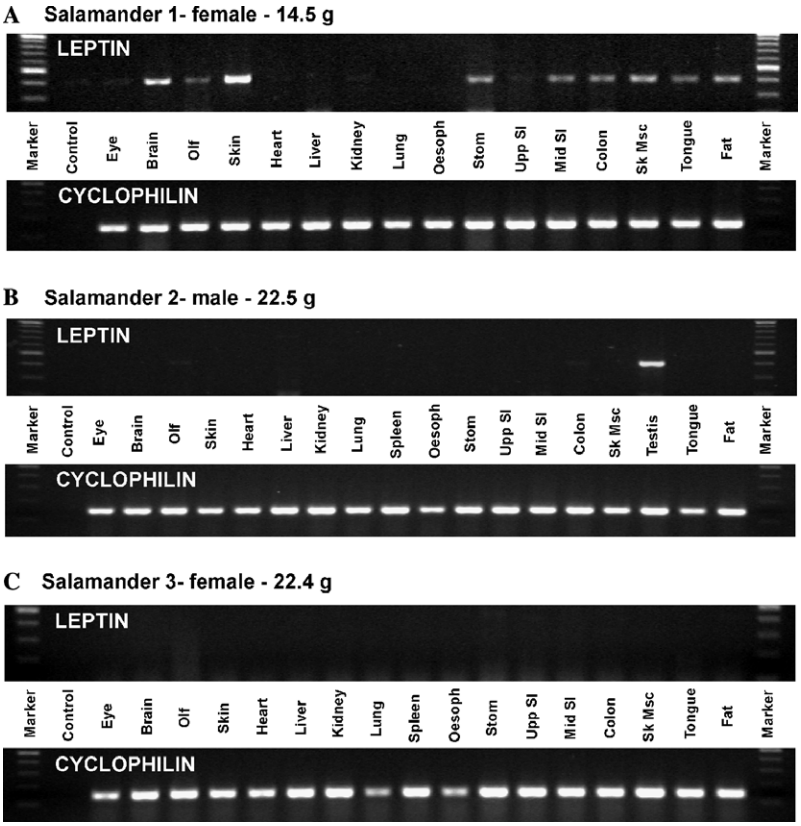

Fig. 7. Tissue distribution of salamander leptin mRNA using RT-PCR. Distribution is shown in three separate individuals (A–C). For each individual, the distribution for leptin mRNA is shown in the upper panel, and for cyclophilin mRNA in the lower panel. Identical lanes were used for leptin and cyclophilin in each individual. Note that the tissues sampled vary slightly between individuals. Also that no leptin gene expression is evident in salamander 3. Tissue abbreviations: Olf, olfactory bulb; Oesoph, oesophagus; Stom, stomach; Upp SI, upper small intestine; Mid SI, mid small intestine; Sk Msc, skeletal muscle; Fat, fat body. Size marker is 100 bp. Control lane contains no template.

PCR product was observed in the skin, with moderate expression also being seen in the brain, stomach, small intestine, colon, skeletal muscle, tongue, and fat body. Weak expression was observed in the olfactory organ. In the two largest animals, a male and a female (Figs. 7B and C), leptin expression was detected only in the testis of the male.

4. Discussion

The results from the present study demonstrate that a sequence found in a salamander EST library is naturally expressed in the tiger salamander and shares structural similarity with mammalian leptin genes. In contrast to the situation with putative chicken leptin cDNAs (Taouis et al., 1998; Ashwell et al., 1999), the demonstration of the existence of an intron in the salamander sequence isolated provides verifiable evidence that this sequence is present in the salamander genome. Moreover, analysis of the rate of non-synonymous substitutions in amphibian, mammal and fish leptin coding sequences is consistent with the accepted consensus of vertebrate evolutionary divergence. In contrast, when the same analysis was performed on the putative chicken leptin coding sequence, the observed rate of substitution was lower than expected statistically (with an associated probability of less than one in one million) causing the chicken to group improbably with rodents in the phylogenetic tree (Dunn et al., 2001).

The predicted amino acid sequence of the salamander protein is consistent with the view that the gene encodes a leptin-like molecule. While the overall amino acid sequence identity between mammalian and salamander leptins is only 29%, the predicted salamander mature leptin-like peptide consists of 146 amino acids as it does in mammals: the two additional amino acids in the full 169-residue salamander sequence are present in the predicted signal peptide, as is also the case with a *Xenopus* sequence. Furthermore, the predicted tertiary structure of salamander leptin conforms to the four-helix bundle structure characteristic of the human protein (Zhang et al., 1997). This is consistent with the predicted salamander protein being a leptin-like molecule and suggests that the residues conserved between the salamander and mammalian leptins are important for generating the tertiary conformation of leptin. In support of this, two cysteine residues required for formation of a disulfide bond

have been conserved in all tetrapod species, and are also present in the recently identified fish leptin-like sequences (Kurokawa et al., 2005). Also, it is noteworthy that 15 of the 48 completely conserved amino acids between the salamander and mammalian leptins are leucine, a residue that is important for the formation of α-helices. The two regions showing the greatest conservation between the salamander and mammals lay between residues 59 and 67 and between residues 102 and 113 and, in terms of molecular structure, these correspond, respectively, to the region between helix A and helix B, and to helix C (Zhang et al., 1997). Comparative molecular evolutionary studies in mammals have highlighted the potential functional importance of residues 85–119 of the mature leptin peptide (equivalent to residues 108–142 in the full salamander sequence, Fig. 3) that includes the end of helix C and the C/D loop with helix E. It has been reported that this region of the leptin molecule is under positive selection in hominoid primates because it shows a greater ratio of non-synonymous to synonymous substitutions when compared with rodents and other mammals (Benner et al., 2002; Gaucher et al., 2003). The findings of the present study are consistent with residues 85–119 showing a high degree of sequence variation between species but the functional implications of this are uncertain. It has been argued that this region of the leptin peptide is critical for induction of appetite suppression and loss of body mass in obese mice, and that the evolutionary changes between positions 85 and 119 in humans may explain the limited therapeutic efficacy of leptin in human clinical trials (Benner et al., 2002; Gaucher et al., 2003). On this basis, given the sequence differences in this fragment between the salamander and rodents, it could be argued that leptin might not be expected to exert potent effects on energy balance in salamanders. However, the consensus from studies of the physiological effects of synthetic leptin peptide fragments in the laboratory mouse and rat is that the most bioactive fragments actually lie between residues 116 and 140, almost completely outside the 85–119 region reported to be under positive selection in hominoids (Lee et al., 2002; Oliveira et al., 2005). It is therefore unlikely that taxonomic sequence variation in this region underlies functional differences in the effects of leptin on energy balance between species.

The similarity of the salamander leptin-like protein sequence to mammalian leptins is matched by a comparable gene structure. Thus, in mammals, the coding sequence of the mature leptin peptide comprises exons 2 and 3 of the leptin gene (He et al., 1995). In the salamander, the coding sequence also contains an intron, in an equivalent position. Analysis of leptin gene structure in *Takifugu* (Kurokawa et al., 2005) indicated the existence of three exons, as in the human gene. This demonstrates that the organisation of the leptin gene has been conserved during vertebrate evolution. The predicted intron size in the salamander is, at approximately 5.5 kb, over three times longer than in the mouse, and this is consistent with the relatively larger size of salamander genomes compared to other vertebrates (He et al., 1995; Voss et al., 2001). The cloning of salamander leptin genomic sequence in the present study provides important evidence that a leptin-like gene is present in the salamander genome.

Although there is evidence for evolutionary conservation of leptin gene structure and conservation of protein motifs, similarity between the leptin-like peptide sequences recently isolated in non-mammalian vertebrates is weak both within and between Classes (Kurokawa et al., 2005). Thus, amino acid sequence similarity between the salamander and *Xenopus* is 60%, and that between the salamander and the four fish leptin sequences ranges from 21 to 25%. Within fish species, sequence similarities lie between 18 and 48% as opposed to the >80% conserved sequence identity found between mammalian leptins (Kurokawa et al., 2005). Leptin-like peptide sequences are therefore markedly different between different fish and amphibian species and this is suggestive of possible taxonomic differences in leptin function. Additionally, the low sequence similarity between fish and amphibian leptin-like peptides and mammalian leptins has implications for the interpretation of studies in which mammalian leptin peptides and antibodies have been applied in non-mammalian vertebrates. Thus, as noted by Kurokawa et al. (2005), leptin-like immunoreactivity reported in studies of fish and amphibians that have used antibodies against mammalian leptins may not actually be specific. For the same reason, the findings of studies in which mammalian leptins have been administered to fish should also be interpreted with caution (Baker et al., 2000; Volkoff and Peter, 2001).

Expression analysis in the present study confirmed that the putative salamander leptin EST sequence represented in the tiger salamander EST library (Putta et al., 2004) is naturally expressed in this species. The leptin-like sequence characterized in the present study shows 98% nucleotide sequence identity with three ESTs obtained independently from the axolotl (Habermann et al., 2004), providing evidence that a similar gene is present in a closely related species. In the tiger salamander, Northern hybridization indicated that the leptin mRNA transcript is approximately 1.9 kb in size. Leptin transcripts vary in size between mammalian species. For example Northern blotting reveals a transcript size of approximately 4.5 kb in the mouse, and 2.3 kb in the little brown bat (*Myotis lucifugus*) (Zhao et al., 2003). Confirmation of the transcript size in the salamander awaits characterization of the 3′-untranslated region of the cDNA, which was not attempted in the present study.

The tissue distribution of salamander leptin-like gene expression differed between the individual

animals studied. Widespread expression was detectable only in the smallest animal and, in the two larger animals, expression was confined to the testis of the male, with no expression being detected in any tissue in the female. To make better comparisons with leptin mRNA distribution in mammals, we chose to focus on adult, metamorphosed, salamanders. The ages of the animals used in the present study are unknown. However, it is known that body size is correlated with age in tiger salamanders and other Urodeles (Hota, 1994; Townes-Anderson et al., 1998). The observation that widespread leptin-like mRNA expression was only detected in the smallest (and therefore youngest) individual that we examined, together with the fact that the leptin-like ESTs represented in the ambystomatid salamander EST databases were obtained from larvae rather than adults (Habermann et al., 2004; Putta et al., 2004) raises the possibility that leptin gene expression may be developmentally regulated in salamanders. The variation in the pattern of leptin-like gene expression between the three individual animals makes it difficult to attach particular importance to expression in specific tissues. However, it is noteworthy that leptin-like mRNA was observed in the fat body, muscle, and in the gut. This is consistent both with reported sites of leptin gene expression in mammals (Bado et al., 1998; Wang et al., 1998; Zhang et al., 1994) and with the presence of leptin mRNA or leptin-like immunoreactivity in the gut of mammals, amphibians, fish and reptiles (Buono and Putti, 2004; Muruzábal et al., 2002). The most intense leptin-like signal intensities were detected in the skin of one salamander and in the testis of another. Leptin expression has been demonstrated in mammalian fibroblasts and the peptide has been linked to a role in wound-healing (Frank et al., 2000; Glasow et al., 2001). Moreover, amphibian skin is well known as being a rich source of bioactive peptides, often with antimicrobial properties (Rinaldi, 2002). Regarding the gonads, leptin expression has not, to our knowledge, been detected in the mammalian testis but the peptide has been implicated in the regulation of testicular steroidogenesis (Caprio et al., 2001). We were unable to confirm whether leptin is also expressed in the salamander ovary because these organs were too small to be dissected in the two females examined. In contrast to the findings of the recent study of fish leptins (Kurokawa et al., 2005), no evidence was found in the present study for leptin-like expression in the liver. This supports the idea that the functions of leptin may differ between vertebrate Classes, as suggested by the marked differences in leptin sequence identity within Classes.

In conclusion, we have characterized a molecule in the tiger salamander genome that shares similarities to mammalian leptins in its nucleotide and amino acid sequence, exon structure, and predicted protein structure. The fact that leptin-like genes have now been identified in amphibians and fish provides important evidence that leptin is of ancient origin, rather than having evolved recently in mammals. This raises intriguing questions about the evolution of the leptin gene in vertebrates. For example, does the apparent absence of leptin genes in vertebrate taxa such as birds suggest that the leptin gene has been deleted from the genome in these groups, or has it yet to be discovered? The present study provides a basis for answering such questions and to explore how the physiological functions of leptin have changed during evolutionary history.

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