# Development and partial characterisation of an antiserum against apolipoprotein B of the short-finned eel, Anguilla australis

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ORIGINAL PAPER

# Development and partial characterisation of an antiserum against apolipoprotein B of the short-finned eel, *Anguilla australis*

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**Abstract** Despite its key role in transportation of triacylglycerides in blood, the distribution, localisation and molecular weight variants of apolipoprotein B (Apob) in teleost fish have essentially escaped study. To address this, a specific short-finned eel (*Anguilla australis*) Apob antiserum was produced by an immunised rabbit, purified and partially characterised. Localisation of Apob at both the mRNA (in situ hybridisation) and protein (immunohistochemistry) levels mirrored that of mammals; thus immunostaining was confined to the interstitial spaces of the liver and the vascular core of the intestinal villi. Immunostaining of proteins by Western blotting, followed by high-resolution LC–MS, indicated that peptide sequence coverage of Apob in low-density lipoproteins spanned the full-length protein. We conclude that only full-length Apob is produced by eels and that both liver and intestine are key sites for its synthesis.

**Keywords** Apolipoprotein B · Antiserum · *Anguilla australis* · In situ hybridisation · Immunohistochemistry

**Abbreviations**

| | |
|---|---|
| 2ME | 2-Mercaptoethanol |
| Apob | Apolipoprotein B |
| BCIP | 5-Bromo-4-chloro-3-indolyl phosphate |
| CBB | Coomassie brilliant blue |
| DEPC | Diethylpyrocarbonate |
| DIG | Digoxigenin |
| Eef1α | Eel elongation factor one alpha |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| LC–MS | Liquid chromatography–mass spectrometry |
| Ldl | Low-density lipoprotein |
| NBT | 4-Nitro blue tetrazolium chloride |
| PBS | Phosphate-buffered saline |
| PFA | Paraformaldehyde |
| PVDF | Polyvinylidene fluoride |
| qPCR | Quantitative polymerase chain reaction |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| tag | Triacylglyceride |
| Vldl | Very low-density lipoprotein |

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## Introduction

The transport of lipids, including triacylglycerides (tags), cholesterol and phospholipids, in blood hinges on the incorporation of these lipids into lipoprotein particles (Spector 1984); these particles are essentially composed of a lipid-rich hydrophobic core and a protein-rich hydrophilic coat (Gibbons 1990). Apolipoprotein B (Apob) is the major lipoprotein associated with all low-density lipoproteins (Segrest et al. 1994). It is vital for the formation of lipoproteins and for their recognition by specific receptors (Weisgraber 1994; Morrow et al. 2000). In mammals, there are two forms of the Apob protein, Apob100 and Apob48 (Chen et al. 1987; Powell et al. 1987). Both proteins are encoded by the same gene, the truncated Apob48 (MW: 240 kDa) sharing the exact same sequence as the N-terminal 48 % of Apob100 (MW: 510 kDa) (Milne et al. 1984; Kovar and Havel 2002).

Whilst it is known that both forms of Apob are synthesised on polysomes bound to the cytoplasmic surface of the endoplasmic reticulum (Boren et al. 1993), it was originally thought that Apob48 was exclusively synthesised in the small intestine (Sorci-Thomas et al. 1989) and Apob100 in the liver (Alexander et al. 1976). This suggested that Apob48 was solely associated with chylomicrons that transported dietary tags from the intestine to the liver (Phillips et al. 1997). Conversely, Apob100 appeared to be associated with very low-density lipoprotein (Vldl) and low-density lipoprotein (Ldl) particles that carried both endogenous and re-packaged dietary tags to various tissues of the body (Bostrom et al. 1988). However, the liver of some mammals (rats and mice) has since been reported to produce Apob48 (Srivastava et al. 1992). To date, debate over the specifics of Apob regulation and the control over which isoform is produced remains, for instance in the context of hormonal or nutritional status.

Whilst both Apob48 and Apob100 have been identified in all previously investigated mammalian species (i.e. platypuses to humans), only the presence of Apob100 has so far been confirmed in any of the lower vertebrate species studied (chicken: Williams 1979; Blue et al. 1980; Tarugi et al. 1990, turtle: Perez et al. 1992; Paolucci and Callard 1995, frog: Paolucci et al. 1998). Varying sizes of Apob have been identified in multiple fish species [rainbow trout (*Oncorhynchus mykiss*) 26, 79, 86, 240, 260 kDa: Chapman et al. 1978; Skinner and Rogie 1978; Babin 1987, Japanese eel (*Anguilla japonica*) 220, 245, 265, and 290 kDa: Yu et al. 1992], yet, it remains unclear whether these correspond to mammalian Apob48 and Apob100. In contrast to most mammals, Apob100 (as well as the many different-sized fragments found in fish) has been identified in both the intestine and the liver in all lower vertebrate species studied to date.

Due to the involvement of Apob in a number of important human pathologies (e.g. atherosclerosis and hyperlipidemia), extensive research has been carried out in this area. However, work in oviparous animals, especially fish, is notably lagging behind which is reflected in the paucity of data and publications. Indeed, the majority of studies in fish have relied on the mobility of apolipoproteins during electrophoresis to evaluate the presence and estimate the molecular weights of the apolipoproteins associated with the various lipoproteins.

Given the importance of lipids in energy metabolism and as determinant of egg quality (Wiegand 1996; Yanes-Roca et al. 2009; Callan et al. 2012), a more detailed understanding of synthesis and the dynamics of Apob is warranted. The present study therefore employed a suite of biochemical techniques with the aim of clarifying the number of Apobs synthesised in short-finned eels and of visualising their localisation (in situ hybridisation and immunohistochemistry). For this purpose, a specific Apob antiserum was generated and employed to identify Apob proteins by Western blotting. Protein identity was then confirmed using liquid chromatography–mass spectrometry. Accordingly, our exploits are the first to produce a specific antiserum for Apob in any species of fish and, when combined with the biochemical follow-up analyses, has provided the first definitive partial characterisation of a fish Apob protein.

## Methods

### Primer design

Two pairs of synthetic oligonucleotide primers were designed to allow (1) quantitative polymerase chain reaction (qPCR) (Apob qPCR FW: GATGTGCCTCAGACATGCAG, Apob qPCR RV: GTATTCTTTGCCATTGCAGCTT) and (2) the production of recombinant protein and probes for in situ hybridisation (Apob recom. FW: AGGGATCCGCCAACGTCCCTGAGA, Apob recom. RV: AGAAGCTTTAGATGGGTGACTCGGCGG). Primers used for qPCR were designed after aligning sequence information gained from Illumina HiSeq transcriptome sequencing (unpublished data) of the liver from a previtellogenic short-finned eel with sequence information from the zebrafish (accession number: XM_689735). An exon boundary was identified using the annotated zebrafish genome and one primer (Apob qPCR RV) was designed to span this region. Primers used for recombinant protein work were also designed using transcriptome sequence information, but with the aim to amplify a partial cDNA at the 5′ end of the *apob* gene. Primers accordingly would amplify the corresponding regions of both Apob100 and Apob48 if present. Solubility was checked by submitting the predicted amino acid sequence into the SOSUI search engine version 1.11 (http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisubmit.html) and molecular weight was calculated using the ExPASy ProtParam tool (http://au.expasy.org/tools/protparam.html). Primers were further designed to be in-frame and included restriction enzyme sites [forward primer: BamH1 (GGATCC); reverse primer: HindIII (AAGCTT)]. The reverse primer also included an in-frame stop codon.

### Tissue collection

One wild-caught female short-finned eel in the perinucleolar stage of oocyte development (body weight 463 g, length 59 cm) was caught in a fyke net in 2010 and euthanised using 0.3 g/l benzocaine (see Lokman et al. 1998 for details on location and capture). The blood was collected from

the caudal vein after transection of the tail and pieces of tissue from an array of different organs were snap frozen and stored at −70 °C until required for molecular analysis. Additional intestine, liver, ovary and spleen tissue were snap frozen and stored at −70 °C in preparation for protein extraction and Western blotting. Intestine, liver and ovary tissue were also fixed in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 9.1 mM $Na_2HPO_4.H_2O$, pH 7.4). Tissue for in situ hybridisation was taken through a gradient of sucrose washes (i.e. 5 % sucrose in PBS for 30 min, 10 % for 60 min, 20 % for 90 min and 30 % for 120 min) then embedded in Tissue-Tek® optimal cutting temperature compound (Sakura Finetek). Tissue for immunohistochemistry was fixed in PFA and transferred to 70 % ethanol until later use.

### Tissue processing for molecular analysis

Total RNA was extracted using TRIzol® (Life Technologies) before 5 μg of RNA was treated with TURBO DNase™ (Life Technologies). Complementary DNA was synthesised from 1 μg of DNase-treated RNA using MultiScribe™ Reverse Transcriptase (50 U/μl: Life Technologies) and subjected to PCR (template: 10 ng, total volume: 30 μl) with BIOTAQ Red DNA Polymerase (0.3 U: Total Lab Biosystems Ltd.) to produce amplicons of 143 bp (qPCR) or 699 bp (recombinant protein and in situ hybridisation).

### Amplicon identification

The amplicons were isolated following gel electrophoresis and ligated into the pGEM T-Easy vector (Promega) and transfected into *Escherichia coli* (XL-1) for amplification as described by Setiawan and Lokman (2010). Plasmid vectors were purified using a NucleoSpin® plasmid kit (Macherey–Nagel—Norrie Biotech) and sent for Sanger sequencing (Allan Wilson Centre, Palmerston North, New Zealand).

### Quantitative polymerase chain reaction

All reactions were performed on an MX-3000P quantitative PCR machine. Complementary DNA samples (1 μl of 10 ng/μl) were run in duplicate using SensiFAST SYBR Lo-ROX kit (Bioline). A housekeeping gene [*eel elongation factor one alpha* (*eef1α*)] was run on the same plate (see Setiawan and Lokman 2010 for primer sequences and standard concentrations) and used semi-quantitatively to normalise copy number data. Annealing for both genes was performed for 10 s at 62 °C and 40 cycles of amplification were completed. A melting curve analysis was then carried out by slowly increasing the temperature from 55 to 95 °C to evaluate sudden changes in fluorescence. In addition to the cDNA samples (tissue distribution) to be analysed, two no-template controls and six duplicate standards ($1 \times 10^{-2}$–$1 \times 10^{-7}$ ng) were added to each 96-well qPCR sample plate. Efficiencies of amplification were 98.4 and 97.3 % for *apob* and *eef1α*, respectively. Stratagene qPCR software was used to transform concentration data into copy number.

### Recombinant protein induction and purification

A cDNA encoding a partial *apob* (699 bp) was amplified from liver by PCR and cloned in-frame into the BamH1 and Hind III sites of a 6xHis-tagged induction vector (pQE30, Qiagen).The construct was transfected into *E. coli* (M15) and protein synthesis was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) in 100 ml of LB medium following the instructions laid out in the Expression Section of the Qiagen QIAexpressionist Handbook for high-level expression and purification of 6xHis-tagged proteins. After induction, the culture was centrifuged for 30 min at 4 °C and 3,500*g*, the supernatant was removed and the remaining bacterial pellet was re-suspended in 30 ml of a urea-based denaturing buffer (10 M Tris, 100 mM $Na_2HPO_4$, 8 M urea, pH 8). Induction was confirmed by 10 % sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and visualised using Coomassie Brilliant Blue (CBB) stain. The sample was then combined with TALON® metal affinity resin (Clontech), washed with urea buffer, transferred into a Ni–NTA agarose gravity-flow polypropylene column (Qiagen), washed again and eluted with a low pH urea-based buffer (10 M Tris, 100 mM $Na_2HPO_4$, 8 M urea, 1 M NaCl, pH 4). Successful purification of recombinant Apob was confirmed after 10 % SDS-PAGE and CBB staining.

### Injection and serum collection

A New Zealand white rabbit was injected with antigen (50 μg) in Freund's complete adjuvant (1:1) followed by subcutaneous immunisations (100 μg) on Days 14 and 28 (1:1). Blood was collected (see Lokman et al. 2010) on Day 21, then every 3 days after that for a total of 4 weeks. Blood was allowed to clot before the serum was aspirated and frozen. A combined aliquot of serum collected on Days 30 and 33 was used for subsequent Western blots and immunohistochemistry.

### Cross-absorption of Apob antiserum with *E. coli* proteins

Insert-free pQE30 was expressed in M15 and the bacterial cell pellet, following centrifugation, re-suspended in urea buffer as above. After 1 h centrifugation at 3,500g and

20 °C, the supernatant was retrieved and dialysed (10 kDa tubing) in PBS for 4 days at 4 °C before being combined with 10 ml of Apob antiserum. Cross-absorption was done by incubation overnight at 4 °C, after which the solution was centrifuged for 1 h at 3,500*g* and 4 °C. The supernatant was then combined with 40 % saturated ammonium sulphate and placed on a stirrer overnight at 4 °C. After a final centrifugation at 3,500g and 4 °C for 30 min, the protein pellet was dissolved in 10 ml of PBS and dialysed in PBS for a further 4 days. The resulting Apob antiserum, now devoid of antibodies cross-reacting with non-target *E. coli* proteins, was then used for Western blotting and immunohistochemistry.

### Western blotting

Frozen tissue samples (ovary, liver, gut and spleen) were homogenised in 1× Complete Mini Proteinase Inhibitor (Roche) in PBS, then centrifuged at 4 °C and 10,000*g* for 60 min. Protein concentration of the supernatant was determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich). Sample buffer, either with or without 2-mercaptoethanol (2ME), was added to each supernatant before 50 µg of each sample was loaded onto one of two identical 10 % SDS-PAGE gels. The separated proteins of one gel were transferred onto a polyvinylidene fluoride (PVDF) membrane by electroblotting at 170 mA for 1 h using an XCell II™ Blot Module (Invitrogen). The membrane-immobilised Apob proteins were incubated with antiserum (1:1,000 dilution) overnight at 4 °C, which was in turn detected using anti-rabbit IgG (1:1,000 dilution) conjugated to alkaline phosphatase (AP) (Roche Diagnostics) at room temperature. Blots were developed by incubating them with 0.5 % 5-bromo-4-chloro-3-indolyl phosphate/0.4 % 4-nitro blue tetrazolium chloride (BCIP/NBT; Sigma-Aldrich) in buffer (50 mM Tris, 150 mM NaCl, pH 9). The separated proteins of the duplicate gel were visualised using CBB stain.

### Isolation of Vldl

Very low-density lipoprotein was separated according to the ultracentrifugation method developed by Havel and colleagues (1955) and modified for use on eel serum by Ando and Matsuzaki (1996). Briefly, serum was overlaid with Vldl density solution (2:1; 1.006 g/ml) and centrifuged at 100,000 rpm and 10 °C for 3 h (Beckman TL-100 ultracentrifuge). The milky, white Vldl fraction was aspirated and dialysed in 1 × PBS overnight at 4 °C.

### Amino acid sequencing to confirm antiserum specificity

A sample of isolated Vldl was loaded into every lane of a 10 % SDS-PAGE gel. Half of the gel was subjected to blotting with anti-Apob and the other half was stained with CBB. Both the stained gel and PVDF membrane were taken to the Centre for Protein Research (Biochemistry Department, University of Otago) where the three immune-positive bands (SDS-PAGE gel) were excised and divided between two samples (i.e. the top two bands could not be separated) and digested with trypsin. The peptides thus obtained were separated using liquid chromatography and the sequences of the trypsin fragments determined by mass spectrometry (LC-LTQ Orbitrap MS).

### In situ hybridisation

A total of 5 µg of purified plasmid containing a 699 bp *apob* sequence was used to synthesise digoxigenin (DIG)-labelled sense and anti-sense cRNA probes with Roche's DIG RNA Labeling Mix. Fixed frozen tissue was cryosectioned (8 µm), mounted onto Superfrost®Plus microscope slides (Thermo Scientific), air dried and then re-fixed in 2 % PFA in PBS. In situ hybridisation was then performed according to a slightly modified method of Braissant and Wahli (1998) and Kusakabe and colleagues (2002). Briefly, sections were washed in 1 × PBS, digested with Proteinase K (Roche 1 µg/ml), re-fixed in 2 % PFA, incubated in 0.1 % active diethylpyrocarbonate (DEPC) (Sigma-Aldrich) in PBS, washed in saline-sodium citrate buffer (750 mM NaCl, 75 mM $Na_3C_6H_5O_7$, pH 7) then hybridised with cRNA probes (approx. 400 ng/section). Hybridised probes were then immunologically detected using a 1:1,000 dilution of anti-DIG-AP Fab fragments (150 U/200 µl) (Roche) and visualised by incubation with NBT/BCIP solution.

### Immunohistochemistry

Fixed tissue was embedded in paraffin, sectioned (8 µm) and mounted onto poly-L-lysine microscope slides (Lab-Serv). Immunohistochemistry was then performed according to an adapted method of Ikeuchi and colleagues (1995). Briefly, sections were deparaffinised, hydrated and then incubated in 0.3 % hydrogen peroxide in methanol for 50 min to inactivate any endogenous peroxidase activity. Non-specific binding of antiserum was blocked by incubation with 10 % skim milk for 2 h before sections were incubated overnight at 4 °C with either a 1:600 dilution of Apob antiserum or 1× PBS (control sections). This was followed by 2 h incubation with anti-rabbit IgG (Nichirei Corporation) and a 45 min incubation with streptavidin–biotin–horse radish peroxidase complex (Nichirei Corporation) according to manufacturer's instructions. Colour development was done using 0.01 % diaminobenzidine in 0.02 % hydrogen peroxide. Sections were then dehydrated and cover-slipped using entellan mountant (Merck).

GATGTGCCTCAGACATGCAGCTACATTGTGCACACCCCCGAGTGCACACTGA
GCGAGGTGTCCGACATCGACCCCGCGGGGCTGCCTGTGTACGGACCTGCTGC
TGGAGCTGATGCTTTCC**AAGCTGCAATGGCAAAGAATAC**

**Fig. 1** Partial *apolipoprotein B* cDNA sequence from short-finned eel, *Anguilla australis*, cloned for use in qPCR analysis. Primer Apob qPCR FW is *underlined* and primer Apob qPCR RV is indicated in *bold*

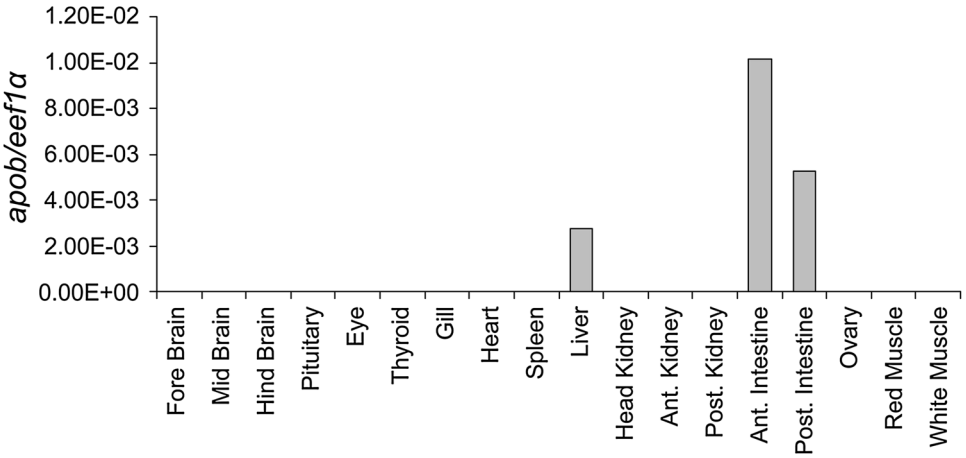


**Fig. 2** Relative transcript copy number of *apolipoprotein B* (*apob*) normalised over *eel elongation factor one alpha* (*eef1α*) in 18 different tissues from a wild-caught female short-finned eel, *Anguilla australis*, in the perinucleolar stage

## Results

### Tissue distribution

A 143 bp fragment of the *apob* gene was successfully cloned (Fig. 1). The relative transcript copy number for *apob* was highest in the anterior intestine, followed by the posterior intestine then the liver (Fig. 2). The relative transcript copy number was very low or undetectable in all other tissues investigated.

### Recombinant Apob and antiserum production

A further 699 bp of the *apob* gene was cloned and submitted to GenBank (Accession number: KF646119). Recombinant Apob production was successfully induced using 1 mM IPTG (Fig. 3a). An initial Western blot showed areas of non-specific binding and background staining in both liver homogenate and induced protein samples (Fig. 3b, lanes 2 and 3, respectively). Absorption with *E. coli* homogenate (pQE30 in M15) greatly reduced both the non-specific binding and the background staining (Fig. 3b, lanes 4 and 5).

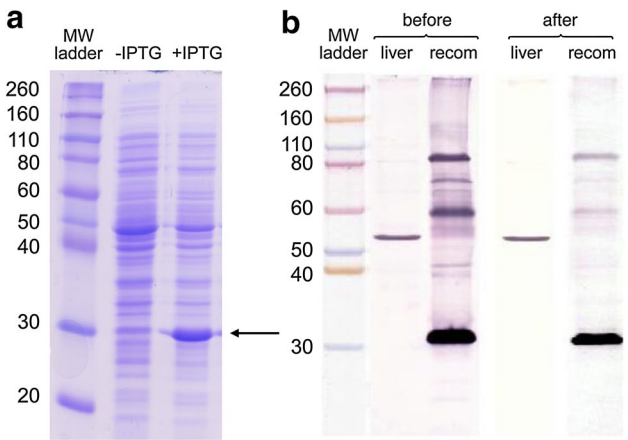


**Fig. 3** 10 % SDS-PAGE gel stained with coomassie brilliant blue (**a**) showing the Novex® sharp pre-stained protein standard with molecular weights (MW) in kDa and recombinant apolipoprotein B (Apob) before and after isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. The *arrow* indicates the induced protein (expected MW = 26 kDa). A PVDF membrane (**b**) incubated with Apob antiserum showing the Novex® sharp pre-stained protein standard, liver homogenate and IPTG-induced recombinant Apob before absorption with *E. coli* homogenate (pQE30 in M15) and liver homogenate and IPTG-induced recombinant Apob after absorption

### Tissue immunoreactivity by Western blot

Three different-sized bands showed positive staining for Apob; the largest stained very weakly, it had a molecular weight higher than 260 kDa, and it remained at the very top of the gel. The second was approximately 110 kDa and the third approximately 55 kDa (Fig. 4b). The different bands appeared in different combinations across the different tissues. The largest band was present in intestine, liver and ovary without 2ME. The smallest band was present

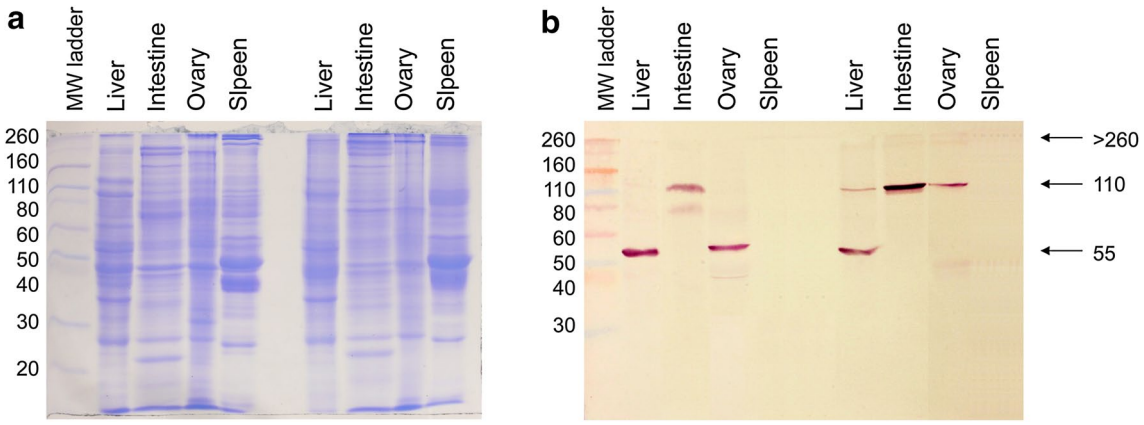


**Fig. 4** 10 % SDS-PAGE gel stained with coomassie brilliant blue (**a**) showing Novex® sharp pre-stained protein standard, and samples of homogenised liver, intestine, ovary and spleen. Samples on the *left* are in buffer containing 2-mercaptoethanol (2ME), samples on the *right* are in buffer without 2ME. The corresponding PVDF membrane after transfer of a duplicate gel (**b**) incubated with Apob antiserum showing the same samples as the 10 % SDS-PAGE gel

in homogenised liver both with and without 2ME and in homogenised ovary with 2ME. The 110 kDa band was present in homogenised intestine both with and without 2ME, and homogenised liver and ovary without 2ME. No positive bands could be seen in homogenised spleen either with or without 2ME.

## Amino acid sequence confirmation

Three bands (approximately 55 kDa and two larger than 260 kDa) showing positive immunoreactivity (Fig. 5b) were chosen for trypsin digest and subsequent amino acid sequencing (the top two bands could not be separated and were combined and sequenced as one band). The total sequencing effort of the trypsin fragments from all bands returned a total of 42 peptides between 9 and 22 amino acids long. Of these 42 peptides, 31 matched either the transcriptome Unigenes that were identified as Apob or corresponded to the Apob sequence from the European eel genome (http://www.zfgenomics.org/sub/eel). The peptides from the largest bands matched regions from throughout the deduced Apob protein although the majority of them were clustered towards the C terminus (Fig. 6). The peptides from the smaller, lighter band which were identified as Apob were solely from the 25 % of the protein closest to the C terminus (Fig. 6). Of the eleven remaining peptides (one from the large bands and ten from the small band), three were identified as different ATP synthase subunits, two as unnamed protein products and the remainder as iron dehydrogenase, cytochrome P-450 lanosterol 14-alpha-demethylase, cysteine dioxygenase, eosinophil peroxidase, macrophage migration inhibitory factor and ubiquitin ligase.

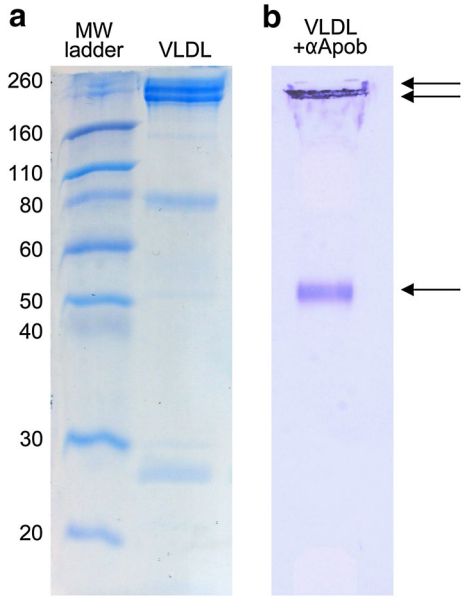


**Fig. 5** 10 % SDS-PAGE gel stained with coomassie brilliant blue (**a**) showing Novex® sharp pre-stained protein standard and very low-density lipoprotein (Vldl) isolated from the serum of a female short-finned eel, *Anguilla australis* in the perinucleolar stage without 2 mercaptoethanol. The corresponding PVDF membrane after transfer from a duplicate gel (**b**) showing a sample of isolated Vldl incubated with apolipoprotein B (Apob) antiserum. Bands highlighted by *arrows* were confirmation sequenced using liquid chromatography–mass spectrometry (LC-LTQ Orbitrap MS) after trypsin digestion

## Apob localisation

*Apob* mRNA was localised within the enterocytes of the intestine (Fig. 7c) and in the cytoplasm of hepatocytes (Fig. 7d). Non-specific staining was not visible on any of the sections incubated with the sense cRNA probe (Fig. 7a, b).


**Fig. 6** Map of amino acid sequencing results. Peptides from sequencing two large bands (>260 kDa) are shown in *grey* and matched regions from throughout the deduced Apob protein. Peptides from the small band (55 kDa) are shown in *black* and cluster in the 25 % of the protein closest to the C terminus

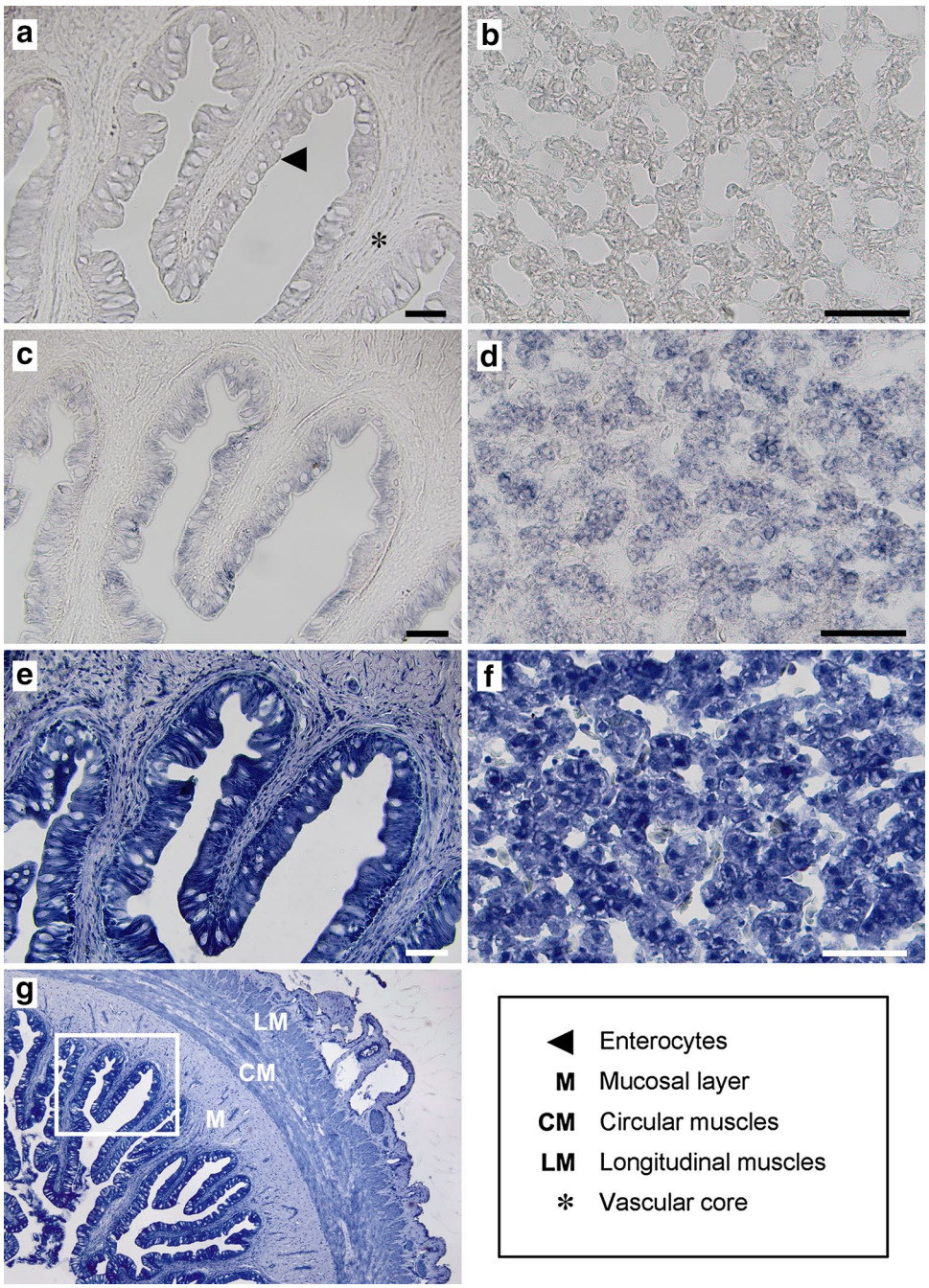


**Fig. 7** Micrographs of sections through the intestine (*left*) and liver (*right*) of a wild-caught female eel, *Anguilla australis* that were incubated with sense (**a** and **b**) or anti-sense (**c** and **d**) apolipoprotein B cRNA probe or stained with haematoxylin (**e**, **f** and **g**). Important morphological features of the intestine are indicated (**g**). *Scale bar* 50 μm

Apob immunoreactivity was localised to the vascular core of the villi of the intestine (Fig. 8c). It was also localised in the sinusoidal spaces of the liver and to a lesser extent in the cytoplasm of the hepatocytes (Fig. 8d). Control slides with either no Apob antiserum (Fig. 8a, b) or no anti-rabbit IgG showed no signal (data not shown).

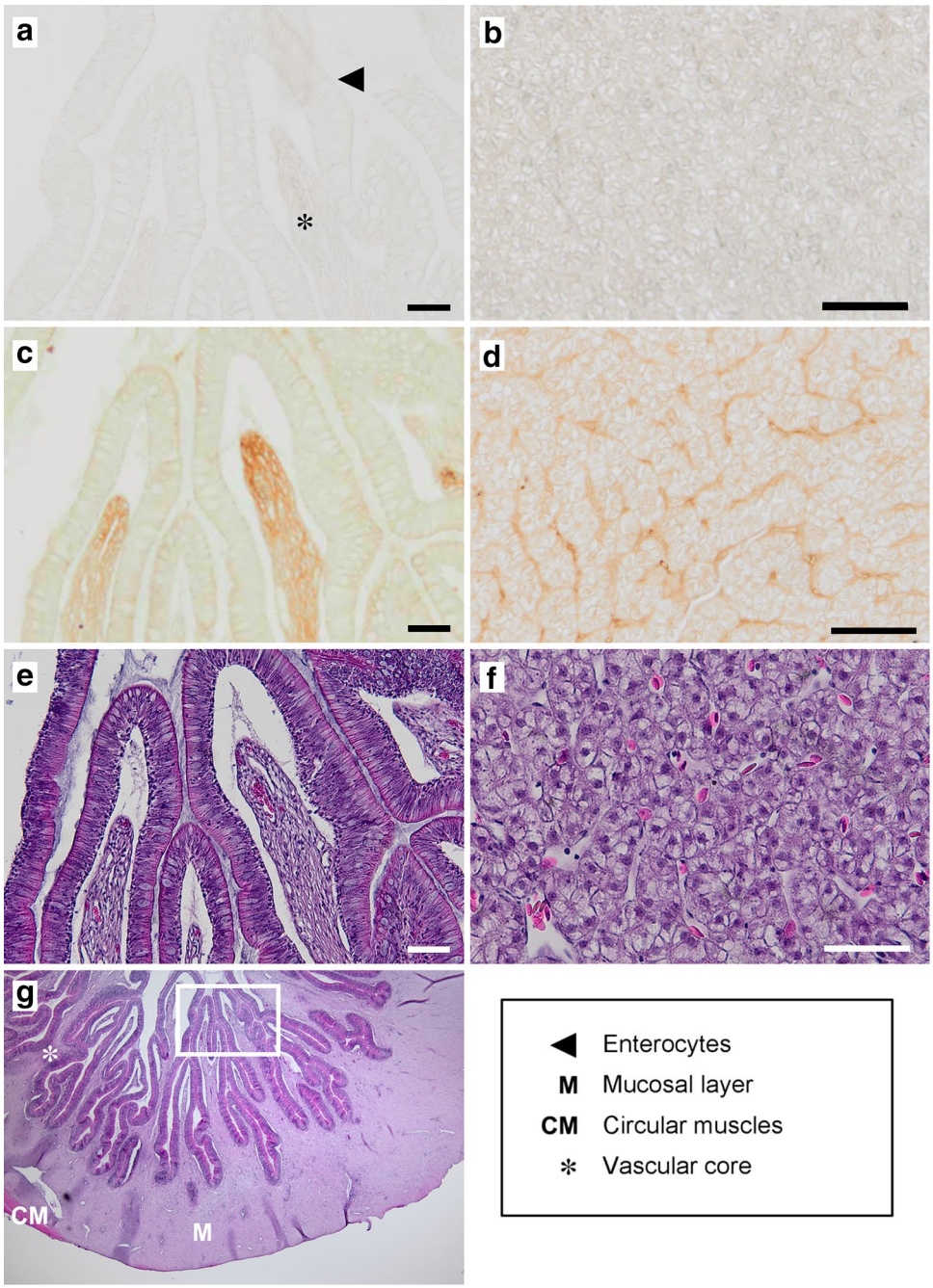


**Fig. 8** Micrographs of sections through the intestine (*left*) and liver (*right*) of a wild-caught female eel, *Anguilla australis*, that were subjected to immunohistochemistry without the primary antiserum (**a** and **b**), immunostained with apolipoprotein B antiserum (**c** and **d**), or stained with haematoxylin and eosin (**e**, **f** and **g**). Important morphological features of the intestine are indicated (**g**). *Scale bar* 50 μm

## Discussion

To synthesise an understanding of lipoprotein physiology in fish, a specific Apob antiserum was developed, characterised and used for immunohistochemistry. Further, *apob* expression was investigated at the mRNA level by identifying which tissues and then which cells, within those positively identified tissues, were synthesising *apob*. These experiments, combined with the use of state-of-the-art biochemical techniques, provide the most compelling evidence to date for the presence of a single *apob* gene in fish.

The molecular weights of human Apob48 and Apob100 have been calculated to be approximately 240 and 510 kDa, respectively (Milne et al. 1984; Kovar and Havel 2002); however, several other smaller fragments have also been identified. These smaller fragments have been shown to be proteolytic fragments generated by kallikrein (Kane et al. 1980; Cardin et al. 1984; Yamamoto et al. 1985) or thrombin (Knott et al. 1985, 1986) cleavage, rather than by mRNA editing. Typically, thrombin and kallikrein digestion results in four fragments (see Cardin et al. 1984), the most common and extensively studied being B74 (MW:

approximately 400 kDa) and B26 (MW: approximately 145 kDa).

The Western blots carried out in this study consistently identified four different-sized bands, two larger than 260 kDa, one around 110 kDa and one 55–60 kDa. It is likely that the band that is larger than 260 kDa corresponds to the full-length Apob100 and may be as large as the mammalian 510 kDa protein (predicted *D. rerio* Apob: 496 kDa, *A. anguilla* genome sequence is incomplete). The fragments of amino acid sequences which resulted from the sequencing of this band aligned throughout the Apob100 sequence, a further indication that this band represents the full-length protein. It is possible that the other band that was sequenced (55–60 kDa) corresponds to either fragment T2 or K4 (of thrombin and kallikrein cleaved Apob100, respectively) as all of the fragments of amino acid sequences resulting from this band align with the C terminus of Apob100. However, the molecular weight of the mammalian T2 and K4 fragments was estimated to be 170 kDa, approximately three times the weight of the immunoreactive small band found in this study. Yet, if the 55–60 kDa band and the 110 kDa band are combined then the resulting molecular weight would approximate that of fragment T2 or K4. The band around 110 kDa was not sequenced; therefore it cannot be determined which fragment it may correspond to. It is also possible that thrombin and/or kallikrein cleavage may produce different-sized fragments in eel. However, a more reasonable explanation arises from previous research, which has shown that, because of the large size of the Apob protein, it is extremely susceptible to extensive degradation and proteolysis by detergents (Steele and Reynolds 1979a, b), oxidative bond cleavage reactions (Schuh et al. 1978) and mechanical shear (Lee et al. 1981) during purification and storage. The detergents used during the SDS-PAGE carried out in this study could therefore have broken the protein. Alternatively, actively forcing such a large protein through a 10 % gel may have resulted in the 55–60 and 110 kDa bands. Indeed, running a 7.5 % SDS-PAGE made little difference to the positioning of the high molecular weight bands but resulted in fewer lower molecular weight bands (data not shown). Without having clean sequence information for the full length of the Apob gene from eels, it is hard to determine the reason for the multiple bands or to confidently identify cleavage sites using sequence information alone. Regardless, this study has found no evidence for the existence of Apob48. Accordingly, it is likely that only Apob100 is present in eels in fact, only Apob100 has been confirmed in other oviparous vertebrates.

Despite potential differences in the number of Apobs, obvious similarities between fish and mammals have arisen from this study. The qPCR results indicate that the only tissues in the eel that are synthesising appreciable amounts of *apob* are the intestine and liver, a finding shared with mammals (Alexander et al. 1976; Sorci-Thomas et al. 1989). These results are supported by those of the in situ hybridisations which showed that *apob* mRNA expression was localised in the enterocytes of the intestinal villi, a localisation identical to that seen in humans (Hopkins et al. 1987; Linton et al. 1991) and mice (Nielsen et al. 1997). Furthermore, the expression of *apob* mRNA in the cytoplasm of the hepatocytes also matches the patterns seen in human liver (Hopkins et al. 1987). The lack of published studies performing in situ hybridisation for *apob* on teleost tissues, or any other oviparous species, makes it hard to draw comparisons. However, several controls were run in this experiment to reduce the possibility of false positive and non-specific staining. Adjacent sections were incubated either without *apob* probe, without anti-DIG-AP Fab fragments or without NBT/BCIP solution and all failed to produce positive staining. In addition, hepatocytes isolated from eels have repeatedly been shown to secrete Apob in in vitro cultures (Yu et al. 1991; Hayashi and Yu 1993; Ndiaye and Hayashi 1997) supporting the validity of the results seen in this study.

Immunohistochemistry showed Apob protein localisation within the sinusoidal spaces of the liver and to a much lesser degree in the cytoplasm of the hepatocytes, an identical expression pattern to what is seen in rat (Davis et al. 1989) and human (Dullaart et al. 1986; Hoeg et al. 1990; Linton et al. 1991; Kjolby et al. 2010). Data in the intestine have been more controversial with some studies reporting protein localisation in the core of the villi, the mucosal layer and others in the enterocytes (Dullaart et al. 1986; Hoeg et al. 1990; Levy et al. 1990). The immunohistochemistry carried out in the present study showed Apob proteins localised within the core of the villi, a position which would presumably facilitate transport.

Interestingly, Apob protein was identified in ovarian homogenates despite *apob* mRNA being almost undetectable in the ovary. This suggests that Apob is being transported into the ovary, possibly via receptor-mediated endocytosis of the low-density lipoproteins (Vldl and Ldl), an interesting result that deserves the attention of future research efforts. The positive immunohistochemical staining for Apob in the ovary (data not shown) confirms the Western blot results and, together with the negative staining in the absence of primary antiserum, adds confidence about the specificity of the antiserum.

The intestine and liver were shown to synthesise Apob in the short-finned eel. Further, a specific antiserum for Apob was successfully produced. The mRNA and protein localisation of these tissues matched the localisation patterns seen in mammals. Although the true molecular weight of full-length Apob in eels is yet to be determined and the reasons for the numerous small fragments identified remain

elusive, this study provides the compelling evidence for the presence of only the full-length *apob* gene in fish.

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**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical standards** The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National University Corporation, Hokkaido University and the animal experiment was performed in accordance with the Hokkaido University manual for implementing animal experimentation.

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