Molecular Characterization and Developmental Expression Pattern of the Chicken Apolipoprotein D Gene: Implications for the Evolution of Vertebrate Lipocalins

María D. Ganfornina,[1*†] Diego Sánchez,[1†] Aldo Pagano,[2,3] Laura Tonachini,[2] Fiorella Descalzi-Cancedda,[4] and Salvador Martínez[5]

The insect Lazarillo and the mammalian apolipoprotein D (ApoD) are orthologous members of the lipocalin protein family. We report the cloning and embryonic expression of chicken ApoD, the first molecularly characterized nonmammalian ApoD. We also report the ApoD expression in mouse during postnatal development and some novel aspects of the expression of the paralogous lipocalin prostaglandin D-synthase (PGDS) and discuss these results in view of the lipocalin family evolution in vertebrates. ApoD is expressed in subsets of central nervous system (CNS) neurons and glia during late chicken embryogenesis. Contrary to mouse ApoD, no expression appears in neural crest-derived cephalic mesenchyme and blood vessel pericytes. Also, ApoD is expressed in developing chicken feathers. These expressions are corroborated by quantitative reverse transcriptase-polymerase chain reaction profiles. ApoD is expressed during mouse postnatal development in a subset of CNS neurons, astrocytes and oligodendrocytes, but also in meninges and pericytes. Chicken PGDS is expressed in brain meninges and perivascular cells. Our results suggest that the amniote last common ancestor expressed ApoD and PGDS in the brain during embryogenesis. ApoD appears restricted to ectodermal derivatives, whereas PGDS is expressed by derivatives of the three germ layers. *Developmental Dynamics 232:191–199, 2005.* © 2004 Wiley-Liss, Inc.

Key words: lipocalin; calycin; neural development; feather development

Received 20 April 2004; Revised 2 August 2004; Accepted 9 August 2004

INTRODUCTION

The nervous systems of flies and grasshoppers benefit from a glycoprotein called Lazarillo for its proper development. Lazarillo belongs to the lipocalin family (Akerstrom et al., 2000a) and is expressed during nervous system development in subsets of neurons and glia, where it appears to have a role in neuronal axon guidance (Sanchez et al., 2000a,b).

Our phylogenetic analyses (Ganfornina et al., 2000) suggest that the protein apolipoprotein D (ApoD) is the vertebrate ortholog of the insect Lazarillo. ApoD is a lipocalin first discovered in the human plasma (McConathy and Alaupovic, 1973) and well studied in mammals (reviewed by Rassart et al., 2000), but its functional role remains to be fully understood.

[1]Departamento de Bioquímica y Fisiología y Genética Molecular-IBGM, Universidad de Valladolid-CSIC, Valladolid, Spain
[2]Istituto Nazionale Ricerca sul Cancro, Genova, Italy
[3]Dipartimento di Oncologia, Biologia e Genetica, Università di Genova/Istituto Nazionale Ricerca sul Cancro, Genova, Italy
[4]Istituto di Bioimmagini e Fisiologia Molecolare, Consiglio Nazionale delle Ricerche, Genova, Italy
[5]Instituto de Neurociencias de Alicante, Universidad Miguel Hernandez-CSIC, Alicante, Spain
Grant sponsor: MCyT; Grant numbers: UE QLRT-1999-31556; UE QLRT-1999-31625; QLRT-2000-02310; DIGESIC-MEC BFI2002-02979; Grant sponsor: REA-Carlos III; Grant number: G03/056.
†Drs. Ganfornina and Sánchez contributed equally to this work.
***Correspondence to: María D. Ganfornina, Departamento de Bioquímica y Fisiología y Genética Molecular-IBGM, Facultad de Medicina, Universidad de Valladolid-CSIC, Avda. Ramón y Cajal 7, Valladolid 47005, Spain. E-mail: opabinia@ibgm.uva.es**

DOI 10.1002/dvdy.20193
Published online 3 December 2004 in Wiley InterScience (www.interscience.wiley.com).

Because of our interest in understanding the function of the Lazarillo-ApoD lipocalin during embryogenesis, we have already studied the expression pattern of ApoD during the embryonic development of the mouse (Sanchez et al., 2002a). However, in contrast to the invertebrate orthologous genes, no neuronal or glial expression of mouse ApoD is detected during the embryonic period analyzed (E8.5–E18.5). Instead, mouse ApoD mRNA is found in the cephalic mesenchyme, meninges, and pericytes surrounding brain and spinal cord capillaries. Variations in expression patterns during the evolution of orthologous genes are not uncommon, and they can help us to identify the biological function of a protein when evolution places it in different cellular contexts or developmental times. Therefore, to fully understand the function of this lipocalin, we set out to search for a functional ApoD gene in another vertebrate model organism, the chicken (*Gallus gallus*), that offers the advantages of well-developed experimental embryology techniques. The orthology between Lazarillo and ApoD predicts the existence of a Lazarillo/ApoD-like protein in the common ancestor of arthropod and chordates and the presence of an ApoD gene in the genome of birds. Nevertheless, the sequence and expression of a chicken ApoD gene has not been reported. An ApoD-like protein was found in chicken blood and oocytes by using immunoblot with an anti-human ApoD antibody (Vieira et al., 1995; Yao and Vieira, 2002), where the authors propose a role for ApoD in the transport of lipids from the yolk to the embryo. However, these results must be taken with caution as they are based on a cross-species immunoreactivity.

In this study, we describe the cloning and sequencing of a cDNA containing the open reading frame (ORF) coding for the entire chicken ApoD protein, demonstrating the presence of a functional ApoD gene in a nonmammalian vertebrate. We analyze the expression profile of chicken ApoD during embryogenesis by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and study its expression pattern by in situ hybridization. This pattern is then compared with the expression of the mouse orthologous gene, mouse ApoD, during postnatal development, which we present in this work as well.

Because of the similar expression of ApoD to the paralogous lipocalin gene prostaglandin D-synthase (PGDS) during embryonic mouse development, we also explore the expression of PGDS in the chicken brain. These results are discussed in view of the evolution of the lipocalin family in vertebrates.

RESULTS AND DISCUSSION

Molecular Cloning and Sequence Analysis of Chicken ApoD

To demonstrate the existence of an avian ApoD, we cloned and sequenced a chicken 225-bp cDNA fragment by using RT-PCR with degenerate primers designed from a mammalian ApoD multiple protein alignment. The sequence of this fragment was used to perform homology searches in the public databases of chicken expressed sequence tag (EST) projects (performed at the Roslin Institute, UK, and the University of Delaware). We found several EST clones that showed exact nucleotide sequence matches with our PCR fragment. Two of these clones (EST ROS068B03 and EST 539b7) were used to assemble a putative chicken ApoD transcript, as they encompassed the longest overlapping cDNA sequence. RT-PCR from chicken Hamburger and Hamilton (HH) stage 39 brain RNA, using unique primers designed from the 5′ and 3′ ends of this conceptual cDNA, provided a single band of the expected sequence (data not shown), that demonstrates the existence of the transcript in chicken embryos.

This transcript bears a single ORF in the 5′-3′ direction that codes for a protein of 189 amino acids. The prediction of a signal peptide and the absence of other strong hydrophobic regions suggest that the transcript translates into a 170 amino acids secreted peptide (Fig. 1A). This mature protein has a molecular mass of 19.85 kDa, and a pI of 5.6. Also, the protein shows two potential N-linked glycosylation sites and a pattern of four cysteine residues (marked in Fig. 1A). The side chains of the putatively glycosylated Asn residues appear accessible in a three-dimensional homology-based model of chicken ApoD (not shown) and, thus, are potential targets to be effectively glycosylated.

BLAST searches using the chicken mature polypeptide revealed significant sequence similarities to mammalian ApoD and its arthropodan relative Lazarillo. Moreover, the inclusion of the chicken protein in an updated phylogenetic analysis of the lipocalin family demonstrates its monophyletic relationship with the Lazarillo–ApoD clade (Fig. 1B). An independent multiple alignment of the chicken ApoD mature protein with selected members of this clade (Fig. 1A) shows several regions of strong similarity in the structurally conserved regions (SCR1–3 in Fig. 1A), a common motif for all lipocalins (Flower et al., 2000) that constitute an essential core for their folding process (Greene et al., 2003). Also, the four cysteine residues that form internal disulfide links in ApoD (Yang et al., 1994) and the grasshopper Lazarillo (Ganfornina et al., 1995) are preserved in all related sequences (asterisks in Fig. 1A), including the new chicken ApoD.

After an extensive BLAST search in the current (June 2004) sequence databases, we performed an ML phylogenetic tree with the complete mature chordate and cephalochordate ApoD proteins recovered from the search. The rooted tree, using the grasshopper Lazarillo sequence as an outgroup protein is shown in Figure 1C. The tree topology parallels the organismal phylogeny, with the chicken ApoD at the base of the land chordates and the amphioxus ApoD at a basal position in the tree. The tree also reveals a gene duplication resulting in up to three paralogous ApoDs in bony fishes. Together, these results suggest that the chicken ApoD transcript encodes a functional ApoD in chicken, which represents the first molecularly characterized nonmammalian ApoD.

Tissue Expression Profile of Chicken ApoD by qRT-PCR

Tissue expression profiles of chicken ApoD were analyzed by real-time qRT-PCR during chicken embryo development (stages HH 39 and 44, respec-

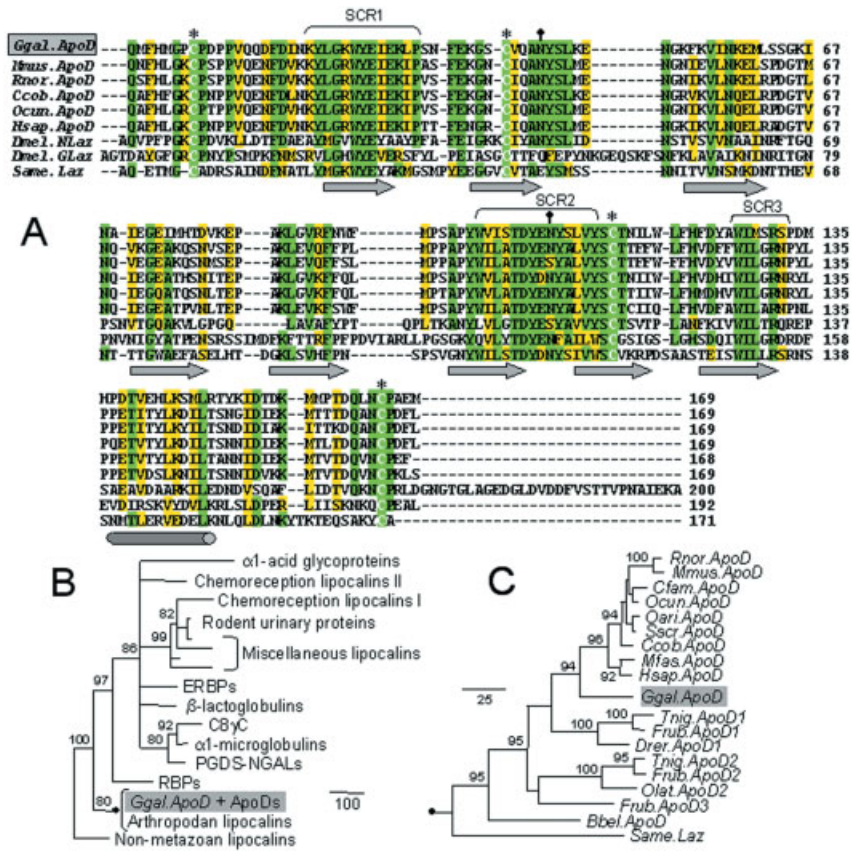


Fig. 1. **A:** The deduced mature protein sequence of the open reading frame present in the chicken apolipoprotein D (ApoD) transcript (GenBank accession no. AY735998) is shown aligned with the mature proteins of selected members of the Lazarillo–ApoD clade. The overall secondary structure of an archetypal lipocalin is shown with β strands represented by arrows and α helices by cylinders. The four conserved cysteines possibly involved in intramolecular disulfide bonds are highlighted by asterisks, and the structurally conserved regions (SCRs) of lipocalins are shown. Potential N-glycosylation sites in chick ApoD are shown with black dots. **B:** ML phylogenetic tree of lipocalins grouped as clades according to node support in the tree. Local bootstrap proportions (LBP) values are indicated at each node. Polytomies reflect nodes with LBP values ≤ 80. The scale bar represents branch length (amino acid substitutions/100 residues). PGDS, prostaglandin D-synthase; RBP, retinol-binding protein; ERBP, epididymal retinoic acid-binding protein; NGAL, neutrophil gelatinase-associated lipocalin. **C:** ML phylogenetic tree of ApoDs based on a distance matrix built upon an alignment of full-length ApoDs revealed by BLAST searches in the current (June, 2004) databases. The tree is rooted with the grasshopper Lazarillo as an outgroup. LBP values are indicated at each node, with a cut-off value ≤ 90. The scale bar represents branch length (amino acid substitutions/100 residues). Proteins are named using an abbreviated species name followed by a functional label: *Rnor*, rat; *Mmus*, mouse; *Cfam*, dog; *Ocun*, rabbit; *Oari*, sheep; *Sscr*, pig; *Ccob*, Guinea pig; *Mfas*: macaque; *Hsap*, human; *Ggal*, chick; *Tnig*, *Tetraodon* pufferfish; *Frub*, *Fugu* pufferfish; *Drer*, zebrafish; *Olat*, medaka fish; *Bbel*, amphioxus; *Same*, grasshopper; Dmel, fruit fly *Drosophila*.

tively) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping control gene. The expression profile of developing chicken embryos at stage HH 39 (Fig. 2A) evidences that chicken ApoD is strongly transcribed in the skin. The chicken ApoD mRNA is 246.48 ± 38.44-fold more abundant in skin than in heart (the sample showing the lowest chicken ApoD transcription level and, thus, used to normalize the expression values). Barely detectable signals were also observed in tibia, brain, and muscle.

A different pattern was observed at stage HH 44, where the brain shows specific chicken ApoD expression along with the skin (Fig. 2B). At this stage, all other tissues investigated show a low level of chicken ApoD synthesis as judged by its mRNA transcription. Therefore, based on this tissue expression profile, we conclude that, at stage HH 39, the chicken ApoD transcription is restricted to the skin, whereas at stage HH 44, the transcription appears in the skin and brain.

Embryonic mRNA Expression Pattern of Chicken ApoD

The clone EST ROS068B03 was used to obtain chicken ApoD riboprobes (566 nucleotides long) for in situ hybridization. These experiments were performed on whole embryos at stages HH 23, 30, and 36 and on selected tissues (liver, heart, brain, and skin) of stage HH 39, 43, and 45 embryos.

In contrast to the strong mRNA expression of mouse ApoD in the embryonic central nervous system (CNS; Sanchez et al., 2002a), chicken ApoD signal was not observed in either the cephalic mesenchymal cells (Fig. 3A), the pericytes of blood vessels, or meningeal cells (Fig. 3B). The first chicken ApoD signal observed in the nervous system appeared at late stages (Fig. 3C–G) in agreement with the qRT-PCR expression profile shown above. The neural ApoD expression is confined to a subset of cells in the brainstem (mainly in the vestibulocochlear complex and the reticular formation, Fig. 3C,G) and the cerebellum and spinal cord (being here restricted to the white matter, Fig. 3C,D,F). These are CNS regions known to express ApoD in other mammals during adulthood (Rassart et al., 2000). A small number of scattered chicken ApoD-positive cells are seen in the thalamus, hippocampus, striatum, and cortex (data not shown). No pericytes, choroidal plexus, or meningeal cells were seen to express chicken ApoD at these late stages of development. No specific signal was detected in liver and heart (not shown).

The qRT-PCR experiments revealed a strong expression of chicken ApoD in the embryo skin (see above). We thus explored the cellular correlate of this expression by in situ hybridization on cryostat sections of HH 43 embryo skin. A specific signal was obtained in developing feathers (Fig. 3H–J). The signal is shown in Figure 3H in a transverse (arrow) and a transverse–sagittal (arrowhead) section of developing feathers at the follicle stage (Yu et al., 2004). No expression was seen in the epidermal or

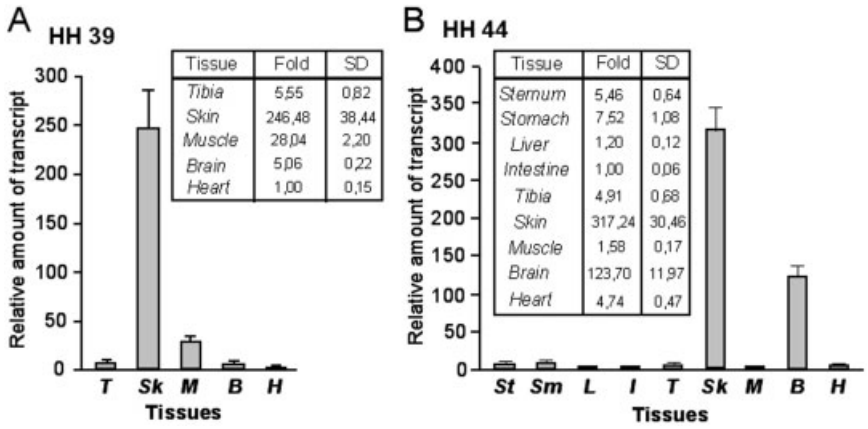


Fig. 2. Chicken ApoD expression profile obtained by real-time quantitative reverse transcriptase-polymerase chain reaction performed on different tissues at two different stages of development. The expression is normalized to that of the glyceraldehyde-3-phosphate dehydrogenase gene and referenced to the tissue with the lowest expression.

dermal skin layers (Fig. 3H). The expression of chicken ApoD was restricted to the marginal plate, at the inner layer of the epidermal collar (Fig. 3J), where a population of cells are undergoing cell death during the process of barb formation (Yu et al., 2004). An epidermal expression of ApoD has also been reported for humans (Zeng et al., 1996) and rodents (Boyles et al., 1990a).

mRNA Expression Pattern of ApoD During the Mouse Postnatal Developmental

Because of the discordant embryonic expression pattern of ApoD observed between mouse (Sanchez et al., 2002a) and chicken (this work), and due to the timing differences in the embryonic development of both organisms (Butler and Juurlink, 1987), we studied the mRNA ApoD expression in the postnatal mouse brain (days P6–P10). The mouse ApoD transcript was found prominently expressed in the cerebellum, brainstem, and spinal cord (Fig. 4A,B), with a pattern similar to that of chicken ApoD during embryonic brain development (see Fig. 3C). Particularly, ApoD-positive cells were observed in the brainstem reticular formation (arrows in Fig. 4A,B). However, in contrast to the chicken, blood vessel pericytes and meningeal ApoD expression was also observed in the mouse postnatal brain (arrowheads in Fig. 4A–C,E). Other cells expressing mouse ApoD were present in the ventral part of the spinal cord (white arrow in Fig. 4B) and along the corpus callosum (arrow in Fig. 4D). Scattered cells were also seen in the thalamus and hippocampus (data not shown), and the striatum (white arrow in Fig. 4D). Something worth noticing is the absence of mouse ApoD or chicken ApoD mRNA expression in Purkinje cells (see Figs. 3D, 4E), which contrasts to the immunohistochemical labeling observed in rodents (Ong et al., 1999). This lack of mRNA expression, also observed in adult mouse Purkinje cells (Sanchez et al., unpublished observations), suggests that in normal conditions ApoD is generated by cerebellar glia and then taken up by the Purkinje neurons.

Cell Types Expressing ApoD in the Chicken and Mouse CNS

Cells expressing the ApoD transcript showed a varied cell body size and morphology in both embryonic chicken and postnatal mouse brains, with small round glial-like cells (arrowheads in Fig. 5A,B) and large polygonal neuron-like cells (arrows in Fig. 5A,B). Although the identity of neural cells expressing ApoD has been reported by using immunohistochemistry with anti-ApoD antibodies in rats (Ong et al., 1999) and humans (Navarro et al., 2004), a colocalization of ApoD mRNA and cell type-specific markers is missing both in mouse and chicken. We performed double in situ immunohistochemistry with antibody markers for neurons (anti-neuronal class III β-tubulin and NeuN), astrocytes (anti–glial fibrillary acidic protein [GFAP]), and oligodendrocytes (anti-PLP). These results are shown in Figure 5C–N.

The location of some chicken and mouse ApoD-expressing cells in the white matter of the cerebellum (Figs. 3D, 4E) suggested a neuroglial character for these cells. The absence of overlapping labeling of ApoD and neuronal markers (Fig. 5C,D) confirms they are neuroglia, in agreement with previous reports in adult rodents (Boyles et al., 1990b; Ong et al., 1999) and primates (Smith et al., 1990; Navarro et al., 2004). On the other hand, the colocalization of the in situ reaction with the expression of neuronal markers in a subset of brain cells (Fig. 5E–G) demonstrates a neuronal expression in both chicken embryos and postnatal mice.

The spinal cord is also a region of strong expression of ApoD in rodents (Boyles et al., 1990b; Seguin et al., 1995). We observed chicken ApoD-positive cells in the spinal cord white

Fig. 3. Chicken apolipoprotein D (ApoD) mRNA expression revealed by in situ hybridizations with a digoxigenin-labeled riboprobe in cryostat sections of chicken embryos. Sections in A–G were counterstained with neutral red. Dorsal is up in all images, unless noted. Developmental Hamburger and Hamilton stages (HH) are indicated in each panel. **A,B:** Chicken ApoD is not expressed in mesenchymal cells (A) and meningeal cells (B). *NE*, neuroepithelium; *Men*, developing meninges; *Mes*, cephalic mesenchyme. **C:** Sagittal section of the brainstem and cerebellum showing chicken ApoD expression. Arrow points to the cerebellar white matter showing specific hybridization. Arrowhead points to brainstem nuclei of the vestibulocochlear complex containing ApoD-positive cells. **D:** A strong ApoD expression is observed the cerebellar white matter (arrow). **E:** Absence of hybridization in the cerebellum with a chicken ApoD sense riboprobe. Open arrowheads in D and E point to the Purkinje cell layer for orientation. **F:** Transverse section of the chicken spinal chord with ApoD expression in cells of the ventral white matter (arrow). The midline is marked by a dotted line. **G:** ApoD-positive cells (arrowheads) surrounding the nucleus magnocellularis (NM) and laminaris (NL) of the cochlear complex. H–J: ApoD expression in the developing feather. **H:** Expression in transversally (arrow) or longitudinally (arrowhead) sectioned feather buds. **I:** Sense probe hybridization shows the specificity of the feather expression in H. **J:** High magnification of a transversal section of a chicken feather follicle showing the cells expressing ApoD located in the marginal plates around the developing barbs.

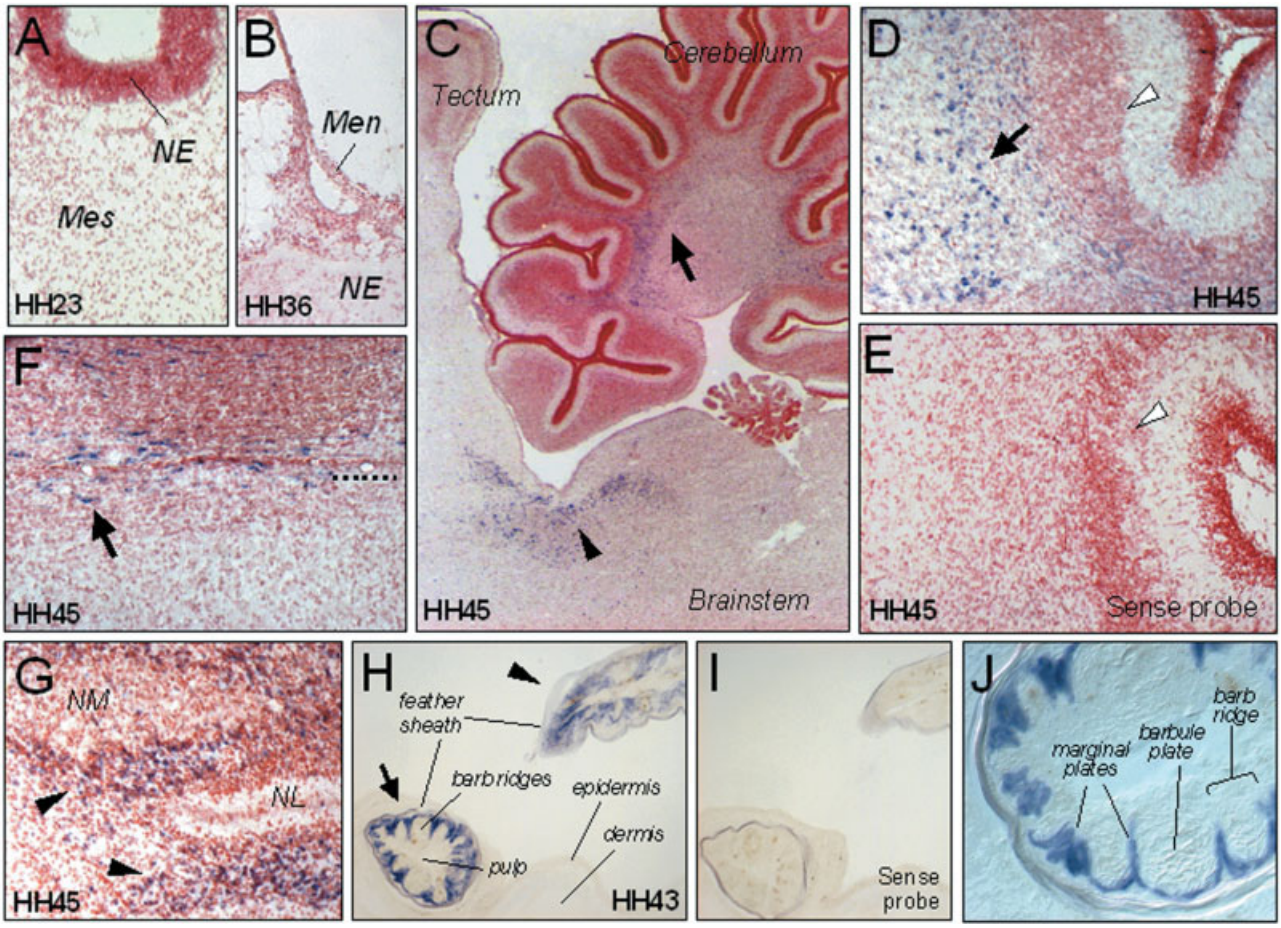


Fig. 3.

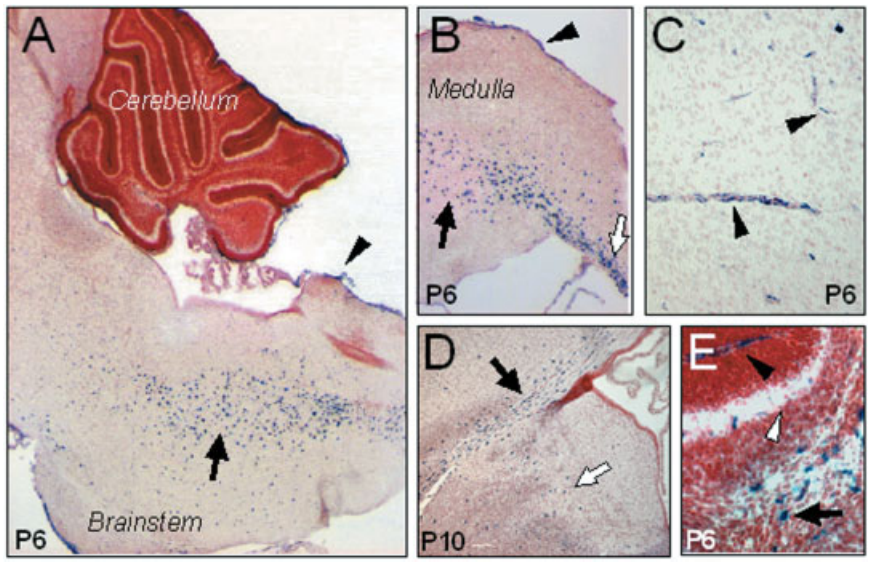


Fig. 4. Apolipoprotein D (ApoD) mRNA expression in mouse during postnatal development. **A,B:** Sagittal sections of a postnatal day 6 (P6) mouse brainstem and cerebellum (A) and medulla and spinal cord (B), showing mouse ApoD expression. Note the expression in meninges (arrowheads) and cells of the reticular formation (arrows in A,B) and ventral spinal cord (white arrow in B). **C:** High magnification of a mouse brainstem area showing ApoD expression in blood vessel pericytes (arrowheads) as well as in scattered neural cells. **D:** Frontal section of the forebrain of a P10 mouse, showing ApoD expression in the corpus callosum (black arrow) and some scattered cells in the striatum (white arrow). The midline is to the right. **E:** Cerebellar expression of mouse ApoD. The arrow points to cerebellar white matter cells. The black arrowhead points to blood vessel labeling in the pial surface, whereas the white arrowhead indicates the Purkinje cell layer. In all images, dorsal is up.

matter (Fig. 5H–J) that showed colocalization of the ApoD transcript with the oligodendrocyte marker PLP (Wight and Dobretsova, 2004). This result demonstrates the expression in oligodendrocytes, a cell type also known to express ApoD in a variety of organisms (Rassart et al., 2000).

The astrocytic identity of some ApoD-positive cells is suggested first by the absence of ApoD expression in the neurons of the nucleus magnocellularis of the cochlear complex (Fig. 5K,L; see also Fig. 3G), where the calretinin-positive neuronal cluster is

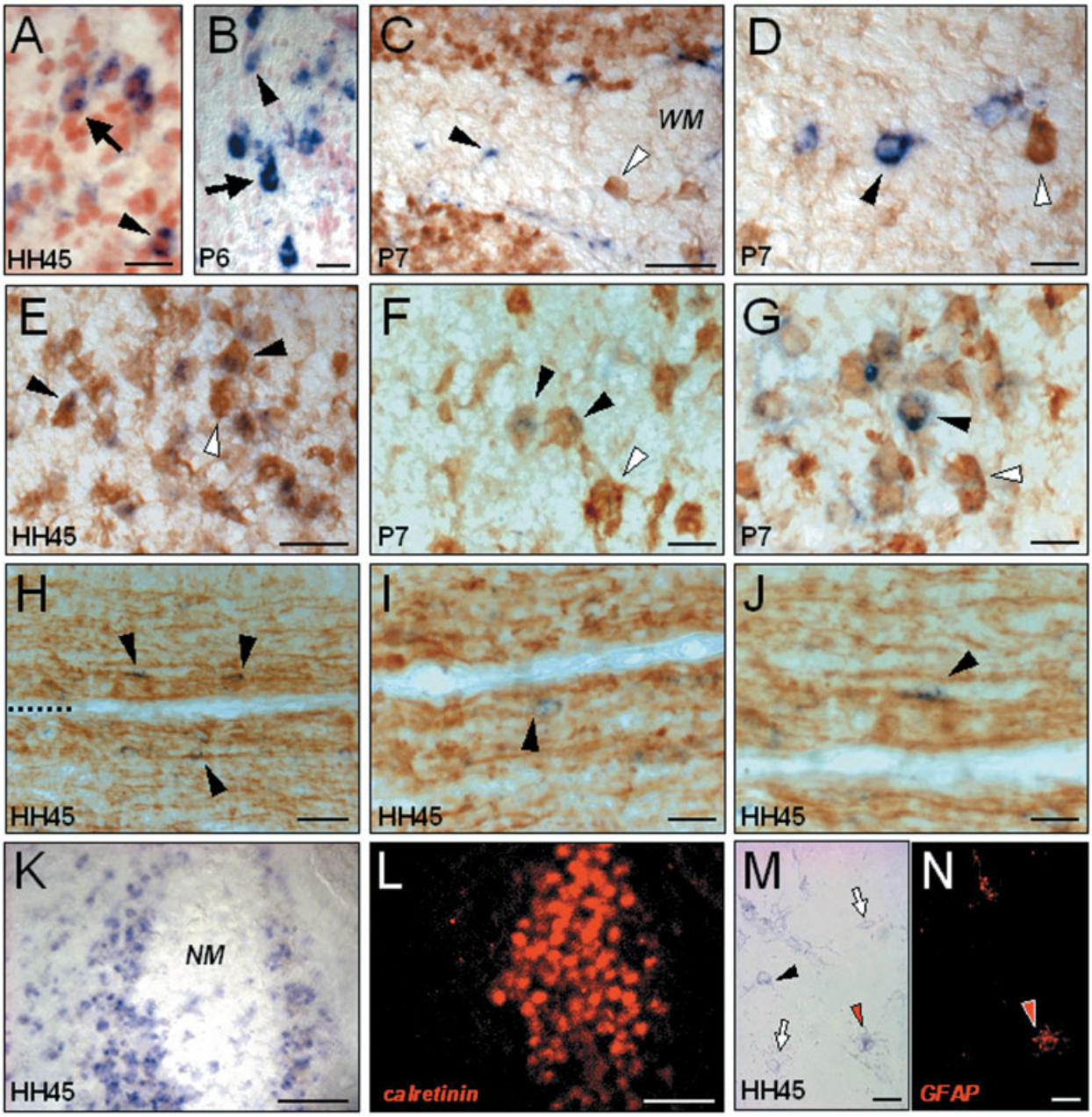
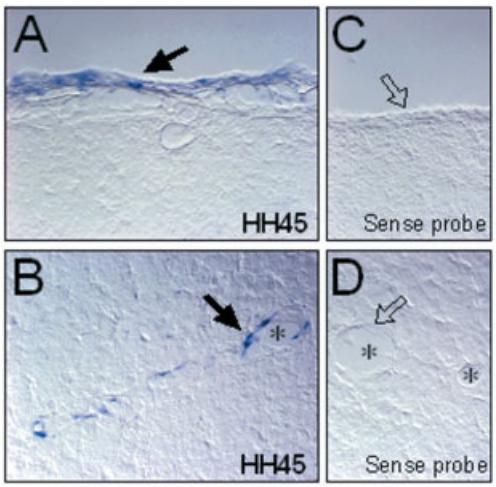


Fig. 5.



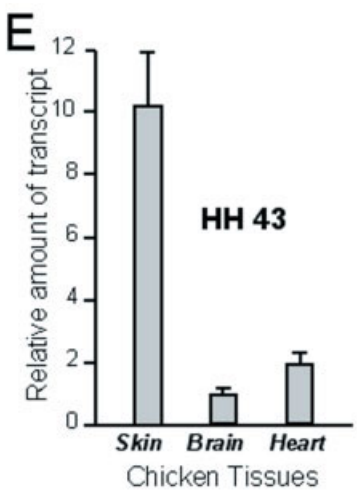


Fig. 6. **A,B:** Prostaglandin D-synthase (PGDS) in situ hybridization in the chicken brain. Arrows point to PGDS-positive cells in the telencephalic meninges (A) and perivascular cells (B). Blood vessel lumen is marked with an asterisk in B. **C,D:** Control labeling using a sense riboprobe. In all images, dorsal is up. **E:** Chicken prostaglandin D-synthase expression obtained by real time reverse transcriptase-polymerase chain reaction in three embryonic tissues (Hamburger and Hamilton stage [HH] 43). The expression is shown normalized to that of glyceraldehyde-3-phosphate dehydrogenase and referred to the tissue with the lowest expression.

surrounded by astrocytes (Kubke and Carr, 2000). Finally, a proof of the astrocytic nature of ApoD-positive cells in chicken is provided by a double in situ immunocytochemistry on primary cultures of embryonic brainstem cells using the astrocyte marker GFAP (Fig. 5M,N). A subset of cells expressing ApoD (arrowheads in Fig. 5M) express GFAP (red arrowhead in Fig. 5M,N). These results agree with a recent colocalization of ApoD protein and GFAP in human brain (Navarro et al., 2004).

ApoD Expression Profile in the Context of Vertebrate Lipocalin Evolution

Our results demonstrate a parallel expression of ApoD in the neuroectodermal derivatives of the CNS in mammals and birds, suggesting the existence of ApoD and its nervous system-specific expression in the last common ancestor of mammals and reptiles. To understand the origin of the mesenchymally derived expression of mouse ApoD during both embryonic and postnatal development, we searched for the expression of other lipocalins related to ApoD. Our phylogenetic analyses suggest an ancient relationship of ApoD with the paralogous lipocalin PGDS. Although the lipocalin retinol-binding protein (RBP) is more similar to ApoD based on protein sequence (see Fig. 1B), RBP shares its main site of expression (liver) with the paralogous lipocalin α1-microglobulin (Akerstrom et al., 2000b; Newcomer and Ong, 2000). On the contrary, PGDS is expressed in the CNS of rodents, specifically in cells of the leptomeninges, the choroid plexuses, as well as in astrocytes, oligodendrocytes, and some neurons of the neural parenchyma (Urade and Hayaishi, 2000) in a pattern resembling that of mouse ApoD. Whether the mesenchymal expression has been co-opted by mammalian ApoD or it was present in the ancestor of mammals and reptiles remains unexplored.

The cloning and tissue expression profile of chicken PGDS has been reported (Pagano et al., 2003) and shows a predominant expression in brain. However, the brain cells expressing the transcript have not been described. Therefore, we studied the PGDS expression pattern in chicken embryonic brain by using in situ hybridization with a PGDS riboprobe. These experiments show specific PGDS expression in chicken developing meninges (Fig. 6A) and blood vessel pericytes (Fig. 6B). However, no neuronal or glial labeling was observed at the stage tested. This pattern bears similarities with that of embryonic mouse brain PGDS expression previously reported (Beuckmann et al., 2000). We also tested the expression of PGDS in chicken skin by qRT-PCR (Fig. 6E) and found evidence of an abundant transcript amount in the dorsal skin of stage HH 43 chicken embryo, the same stage at which chicken ApoD is expressed in developing feathers.

Conclusions

The results presented in this work demonstrate the expression of chicken ApoD in ectodermal derivatives during development. Chicken ApoD is expressed in the developing feather follicles and the nervous system. Both neurons and glia express chicken ApoD in a subset-dependent form, although very dynamic temporal changes of expression cannot be ruled out. By contrast, chicken ApoD was not found in pericytes and meningeal cells, as had been reported in mouse development. Also, a subset of neurons and glia are expressing ApoD in the mouse brain during postnatal development, as well as meningeal cells and some pericytes.

The expression of the paralogous lipocalin gene PGDS suggests that the common ancestor of birds and mammals might have expressed ApoD and PGDS in both mesenchymal and neuroectodermal derivatives. The expression profiles possibly changed after the split of mammals and birds, being chicken ApoD restrictedly expressed by neuroectodermal derivatives.

The discovery of an avian ApoD gene pushes the evolutionary presence of this lipocalin back to the beginning of the amniote radiation some 250 My ago. These results, along with our phylogenetic analyses and the finding of ApoD-like sequences in fishes and cephalochordates (see Fig. 1C), support an ancestral origin of ApoD in the chordate lineage. Finally, the protein sequence relationship of ApoD and the arthropodan Lazarillo and their shared neural expression pattern suggest that the nervous system was part of the expression domain of the lipocalin present in the common ancestor of arthropods and chordates.

Fig. 5. Apolipoprotein D (ApoD) -expressing cell types in the chicken and mouse central nervous system. **A:** High magnification of chicken brainstem cells expressing ApoD. The arrowhead points to a small glial-like cell, and the arrow points to a neuron-like cell. HH, Hamburger and Hamilton stage. **B:** High magnification of cells in the reticular formation, showing the cell-type diversity of mouse ApoD expression. Glia-like (arrowhead) and neuron-like (arrow) cells appear labeled with the antisense riboprobe. P, postnatal day. C–G: Digoxigenin (Dig) -riboprobe in situ hybridization (blue labeling, examples pointed by black arrowheads) of ApoD and neuronal horseradish peroxidase (HRP) immunohistochemistry (brown labeling, examples pointed by white arrowheads) with a combination of anti-class III β-tubulin and anti-NeuN antibodies. **C:** ApoD-positive cells in the white matter of mouse cerebellum. **D:** Brainstem cells expressing ApoD in the mouse. **E:** Chicken brainstem neurons, some with ApoD-specific labeling. **F,G:** Mouse reticular formation neurons, some expressing ApoD. H–J: Dig-riboprobe in situ hybridization (blue labeling, examples pointed by arrowheads) of ApoD and oligodendroglial HRP immunohistochemistry (brown labeling) with anti-PLP antibody. **H:** Transverse section of chicken spinal cord (midline marked by dashed line), showing ApoD-positive cells and oligodendroglial fibers. **I,J:** High magnification of ApoD-expressing cells also expressing PLP. **K,L:** Dig-riboprobe in situ (blue labeling) of ApoD and neuronal immunofluorescence with anti-calretinin antibody in the nucleus magnocellularis (NM) of the cochlear complex. **M,N:** Primary cell culture of brainstem embryonic chicken cells labeled with a dig-riboprobe in situ (blue labeling) for ApoD and astrocyte immunofluorescence with anti–glial fibrillary acidic protein antibody. Cells not expressing ApoD are marked by white arrows. Arrowheads point to ApoD-positive cells. The red arrowheads point to an ApoD-positive astrocytes. Scale bars = 20 μm in A,B,D,F,G,I,J,M,N, 50 μm in C,E,H, 100 μm in K,L.

EXPERIMENTAL PROCEDURES

Amplification and Cloning of the Chicken ApoD Gene

Total RNA was purified from the brain of chicken embryos (stage HH

45) by using Trizol (Invitrogen). One microgram of total RNA was reverse transcribed with MuLV-reverse transcriptase (PE Biosystems) using random hexamers.

PCR amplifications were performed in a thermal cycler (GeneAmp 9700 Perkin Elmer) using thin walled plastic tubes; PE Biosystems). Primers were designed from the conceptual chicken ApoD cDNA sequence (see below) with the primer3 program (www-genome.wi.mit.edu/cgi-bin/primer/primer3_WWW.cgi) and synthesized by Genotek. PCR products were sequenced with an ABI Prism 377 automated DNA sequencer using Taq FS DNA Polymerase.

Real-Time qRT-PCR

Total RNA was extracted from tissues of several embryo stages by using the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). The levels of chicken ApoD mRNA were measured by real-time qRT-PCR using the PE ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Expression of the gene GAPDH (accession no. K01458) was measured as an endogenous control. The sequences of forward and reverse primers, and of a TaqMan fluorogenic probe for chicken ApoD were designed by the Primer Express 1.5 software and are as follows: Forward primer, 5′-ACCCACCAGTACAGCAGG; Reverse primer, 5′-GCAGCTTTTCTATCTCGTAC; Probe, 5′[FAM]-TTCGACATCAATAATTACTTGGGAAA-[TAMRA]3′.The primers and probes for chicken GAPDH and PGDS genes are those described previously (Pagano et al., 2003). The chicken ApoD probe was located at the junction between two predicted exons in the lipocalin genes based on an exon – intron alignment of lipocalins (Sanchez et al., 2003). The threshold cycle (Ct), which correlates inversely with the target mRNA levels, was measured during PCR amplification as the cycle number at which the reporter fluorescent emission increases above a threshold level. Relative transcript levels were determined from the relative standard curve built from stock cDNA dilutions, and divided by the target quantity of the calibrator according to the manufacturer's instructions.

In Situ Hybridization and Immunohistochemistry

Digoxigenin-labeled riboprobes (sense and antisense) of the chicken ApoD transcript comprise the complete coding sequence and part of the 5′-untranslated region. Riboprobes for the chicken ApoD or mouse ApoD transcript were prepared as previously described (Sanchez et al., 2002a). Digoxigenin-labeled sense and antisense of the chicken PGDS transcript were prepared according to Pagano et al. (2003).

Postnatal Swiss-Webster mice (P6–P10) were anesthetized with chloroform, and fixed by transcardial perfusion with 4% paraformaldehyde. Chicken embryos of the selected stages were fixed in 4% paraformaldehyde and stored in methanol at −70°C until processing. The procedures for animal use were approved by the UMH Animal Care Committee. The embryos or the selected tissues were washed in PBS, cryoprotected in sucrose (20% in PBS), mounted in TissueTek (Sakura), and frozen in liquid nitrogen.

In situ hybridizations on 30-μm cryostat sections were performed at 63°C for 18 hr in a hybridization oven as described (Sanchez et al., 2002a). The riboprobes were used at 1 μg/ml. Nonspecific hybridizations were assessed with the sense probes. The hybridized sections were counterstained with neutral red, dehydrated, and mounted with Eukitt. The preparations were observed with a Leica microscope, photographed with a CoolSnap (Photometrics) camera, and processed with Adobe Photoshop.

Immunohistochemistry experiments were performed on 10-μm cryostat sections of chicken embryos and mouse postnatal brains. Blocking and washing solutions were based on a phosphate buffer and contained Triton X-100 (0.1%) as a detergent. Cell type antibody markers were anti-calretinin (Swiss antibodies), anti-class III β-tubulin (Covance), anti-NeuN (Chemicon), anti-PLP (a gift from Dr. B. Zalc), and anti-GFAP (Sigma). Biotin-conjugated secondary antibodies, either in combination with streptavidin-rhodamine (Vector) or with the ABC-horseradish peroxidase kit (Vector), were used to visualize the immunohistochemical reaction.

Cell Culture of Chicken Brainstem Cells

Embryonic (HH 45) brainstems were removed, and cell cultures were prepared as described by Garrido et al. (1998) by tissue dissociation after 0.05% trypsin treatment. Brainstem cells were plated onto poly-L-lysine treated coverslips and cultured in DMEM-modified medium with 1.25% serum. After 36 hr of culture, the cells were fixed in 4% formaldehyde, 5% acetic acid, and 0.9% NaCl. Cell processing for double in situ hybridization immunocytochemistry is described in Sanchez et al. (2002b).

Sequence Analysis, Alignments, and Phylogeny Inference

Sequence analysis of the novel chicken ApoD cDNA (GenBank accession no. AY735998) was carried out by using programs available at the ExPASy Web site. Multiple alignments of selected lipocalin sequences were performed with Clustal X (1.8; Thompson et al., 1997) using a Gonnet series scoring matrix. Phylogenetic analyses of protein sequences were carried out by using a ML-based method, and the tree reliability was evaluated by bootstrapping with 1,000 replicates (Ganfornina et al., 2000).

ACKNOWLEDGMENTS

We thank M. Ródenas and G. Fernández for experimental assistance. We thank C. Santiago at the Centro de Investigaciones Biológicas sequencing facility in Madrid. M.D.G. and D.S. were funded by MCyT start-up grants, and S.M. was funded by REA-Carlos III.

REFERENCES

Akerstrom B, Flower DR, Salier J-P. 2000a. Lipocalins: unity in diversity. Biochim Biophys Acta 1482:1–8.

Akerstrom B, Logdberg L, Berggard T, Osmark P, Lindqvist A. 2000b. alpha(1)-Microglobulin: a yellow-brown lipocalin. Biochim Biophys Acta 1482:172–184.

Beuckmann C, Lazarus M, Gerashchenko D, Mizoguchi A, Nomura S, Mohri I, Uesugi A, Kaneko T, Mizuno N, Hayaishi O,

Urade Y. 2000. Cellular localization of lipocalin-type prostaglandin D synthase (beta-trace) in the central nervous system of the adult rat. J Comp Neurol 428: 62–78.

Boyles JK, Notterpek LM, Anderson LJ. 1990a. Accumulation of apolipoproteins in the regenerating and remyelinating mammalian peripheral nerve. Identification of apolipoprotein D, apolipoprotein A-IV, apolipoprotein E, and apolipoprotein A-I. J Biol Chem 265:17805–17815.

Boyles JK, Notterpek LM, Wardell MR, Rall SC Jr. 1990b. Identification, characterization, and tissue distribution of apolipoprotein D in the rat. J Lipid Res 31: 2243–2256.

Butler H, Juurlink BHJ. 1987. An atlas for staging mammalian and chick embryos. Florida: CRC Press Inc.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Flower DR, North ACT, Sansom CE. 2000. The lipocalin protein family: structural and sequence overview. Biochim Biophys Acta 1482:9–24.

Ganfornina MD, Sánchez D, Bastiani MJ. 1995. Lazarillo, a new GPI-linked surface lipocalin, is restricted to a subset of neurons in the grasshopper embryo. Development 121:123–134.

Ganfornina MD, Gutiérrez G, Bastiani MJ, Sánchez D. 2000. A phylogenetic analysis of the lipocalin protein family. Mol Biol Evol 17:114–126.

Garrido JJ, Alonso MT, Lim F, Carnicero E, Giraldez F, Schimmang T. 1998. Defining responsiveness of avian cochlear neurons to brain-derived neurotrophic factor and nerve growth factor by HSV-1-mediated gene transfer. J Neurochem 70:2336–2346.

Greene LH, Hamada D, Eyles SJ, Brew K. 2003. Conserved signature proposed for folding in the lipocalin superfamily. FEBS Lett 553:39–44.

Kubke MF, Carr CE. 2000. Development of the auditory brainstem of birds: comparison between barn owls and chickens. Hear Res 147:1–20.

McConathy W, Alaupovic P. 1973. Isolation and partial characterization of apolipoprotein D: a new protein moiety of the human plasma lipoprotein system. FEBS Lett 37:178–182.

Navarro A, del Valle E, Tolivia J. 2004. Differential expression of apolipoprotein D in human astroglial and oligodendroglial cells. J Histochem Cytochem 52: 1031–1036.

Newcomer ME, Ong DE. 2000. Plasma retinol binding protein: structure and function of the prototypic lipocalin. Biochim Biophys Acta 1482:57–64.

Ong WY, Lau CP, Leong SK, Kumar U, Suresh S, Patel SC. 1999. Apolipoprotein D gene expression in the rat brain and light and electron microscopic immunocytochemistry of apolipoprotein D expression in the cerebellum of neonatal, immature and adult rats. Neuroscience 90:913–922.

Pagano A, Crooijmans R, Groenen M, Randazzo N, Zerega B, Cancedda R, Dozin B. 2003. A chondrogenesis-related lipocalin cluster includes a third new gene, CALgamma. Gene 305:185–194.

Rassart E, Bedirian A, Do Carmo S, Guinard O, Sirois J, Terrisse L, Milne R. 2000. Apolipoprotein D. Biochim Biophys Acta 1482:185–198.

Sanchez D, Ganfornina MD, Bastiani MJ. 2000a. Lazarillo, a neuronal lipocalin in grasshoppers with a role in axon guidance. Biochim Biophys Acta 1482:102–109.

Sanchez D, Ganfornina MD, Torres-Schumann S, Speese SD, Lora JM, Bastiani MJ. 2000b. Characterization of two novel lipocalins expressed in the Drosophila embryonic nervous system. Int J Dev Biol 44:349–360.

Sanchez D, Ganfornina MD, Martinez S. 2002a. Expression pattern of the lipocalin apolipoprotein D during mouse embryogenesis. Mech Dev 110:225–229.

Sanchez D, Lopez-Lopez JR, Perez-Garcia MT, Sanz-Alfayate G, Obeso A, Ganfornina MD, Gonzalez C. 2002b. Molecular identification of Kvalpha subunits that contribute to the oxygen-sensitive K+ current of chemoreceptor cells of the rabbit carotid body. J Physiol 542:369–382.

Sanchez D, Ganfornina MD, Gutiérrez G, Marín A. 2003. Exon-intron structure and evolution of the lipocalin gene family. Mol Biol Evol 20:775–783.

Seguin D, Desforges M, Rassart E. 1995. Molecular characterization and differential mRNA tissue distribution of mouse apolipoprotein D. Brain Res Mol Brain Res 30:242–250.

Smith KM, Lawn RM, Wilcox JN. 1990. Cellular localization of apolipoprotein D and lecithin:cholesterol acyltransferase mRNA in rhesus monkey tissues by in situ hybridization. J Lipid Res 31:995–1004.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. Nucleic Acids Res 24:4876–4882.

Urade Y, Hayaishi O. 2000. Biochemical, structural, genetic, physiological, and pathophysiological features of lipocalin-type prostaglandin D synthase. Biochim Biophys Acta 1482:259–271.

Vieira AV, Lindstedt K, Schneider WJ, Vieira PM. 1995. Identification of a circulatory and oocytic avian apolipoprotein D. J Mol Reprod Dev 42:443–446.

Wight PA, Dobretsova A. 2004. Where, when and how much: regulation of myelin proteolipid protein gene expression. Cell Mol Life Sci 61:810–821.

Yang CY, Gu ZW, Blanco-Vaca F, Gaskell SJ, Yang M, Massey JB, Gotto AM, Pownall HJ. 1994. Structure of human apolipoprotein D: locations of the intermolecular and intramolecular disulfide links. Biochemistry 33:12451–12455.

Yao Y, Vieira A. 2002. Comparative 17beta-estradiol response and lipoprotein interactions of an avian apolipoprotein. Gen Comp Endocrinol 127:89–93.

Yu M, Yue Z, Wu P, Wu D, Mayer JA, Medina M, Widelitz RB, Jiang T, Chuong C. 2004. The developmental biology of feather follicles. Int J Dev Biol 48:181–191.

Zeng C, Spielman AI, Vowels BR, Leyden JJ, Biemann K, Preti G. 1996. A human axillary odorant is carried by apolipoprotein D. Proc Natl Acad Sci U S A 93:6626–6630.