

A chondrogenesis-related lipocalin cluster includes a third new gene, CAL γ

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Received 16 September 2002; received in revised form 16 December 2002; accepted 31 December 2002

Received by R. Di Lauro

Abstract

We have previously reported the modulation, during chondrogenesis and/or inflammation, of two chicken genes laying in the same genomic locus and coding for two polypeptides of the lipocalin protein family, the extracellular fatty acid binding protein (ExFABP) and the chondrogenesis associated lipocalin β (CAL β). A third gene, located within the same cluster and coding for a new lipocalin, CAL γ , has been identified and is here characterized. Tissue distribution analyzed by real-time quantitative reverse transcriptase-polymerase chain reaction in chicken embryos shows a ubiquitous expression with predominant levels of mRNA transcripts in the liver and the brain. In the developing tibia, a high expression of CAL γ mRNA was evidenced by in situ hybridization within the pre-hypertrophic and the hypertrophic zones of the bone-forming cartilage. In agreement, dedifferentiated chondrocytes in vitro express the transcripts to the highest level when they re-differentiate reaching hypertrophy. Such peculiar developmental pattern of expression that is analogous to those already described for ExFABP and CAL β suggests that all three proteins may act synergistically in the process of endochondral bone formation. Moreover, like ExFABP and CAL β , CAL γ is also highly induced in dedifferentiated chondrocytes upon stimulation with lypopolysaccharides, indicating that the whole cluster quite possibly is transcriptionally activated not only in physiological morphogenic differentiation but also in pathological acute phase response.

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Keywords: Chondocyte differentiation; Extracellular matrix; Inflammation

1. Introduction

Lipocalins are a protein family whose members share a β -barrel secondary structure that creates their peculiar pocket-like folding. Based on this characteristic, the lipocalin family is in turn part of the larger calycin superfamily that comprises other proteins with a similar

tertiary structure (Flower et al., 1993; Flower, 1996). The lipocalins also share several biochemical features such as a diagnostic amino-acid motif (the so called 'lipocalin domain'), eight anti-parallel β -sheets (that make up the β -barrel folding), three α -helices and a tryptophan in the N-terminal half of the primary sequence (Flower et al., 1993, 2000). Although lipocalins are known to be present at almost every evolutionary level, from bacteria to mammals, function and mechanism of action have been clarified for only some of them. Prostaglandin D synthase (Nagata et al., 1991; Peitsch and Boguski, 1991), violaxanthin de-epoxidase and zeaxanthin epoxidase (Bugos et al., 1998; Hieber et al., 2000) have specific enzymatic functions. Other lipocalins have been related to a wide range of different biological processes among which cell homeostasis, pro-

Abbreviations: aa, aminoacid; bp, base pair; cDNA, complementary DNA; CAL β , chondrogenesis associated lipocalin beta; CAL γ , chondrogenesis associated lipocalin gamma; ExFABP, extracellular fatty acid binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Kb, kilobase; LPS, lipopolysaccharides; mRNA, messenger RNA; nt, nucleotide; RT-PCR, reverse transcriptase-polymerase chain reaction.

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liferation and differentiation (Flower, 1994), growth and repair within the nervous system (Sanchez et al., 2000), chemosensory signaling (Cavaggioni and Mucignat-Caretta, 2000), fertility and reproduction (Halttunen et al., 2000), acute systemic inflammation and immunomodulation (Logdberg and Wester, 2000) and tumor invasion (Bratt, 2000). Still, due to their tertiary structure, most of them are thought to serve as carriers of specific hydrophobic molecules as retinoids, fatty acids, cholesterol, prostaglandins, pheromones and odorants (Flower, 1996).

In previous reports, we have characterized two lipocalins specifically associated with the process of chondrocyte differentiation, the extra-cellular fatty acid binding protein (Cancedda et al., 1990) and the chondrogenesis associated lipocalin β (CAL β) (Pagano et al., 2002). Indeed, both proteins are abundantly synthesized and secreted by fully mature hypertrophic chondrocytes while only traces of mRNA transcripts and/or proteins are detected in dedifferentiated proliferating precursor cells. They are also similarly up-regulated in response to inflammatory stimulation with lipopolysaccharides (LPS) (Cermelli et al., 2000; Pagano et al., 2002). At a genomic level, both genes are organized in tandem within the same chromosomal locus, thus potentially forming a lipocalin cluster. This notion of cluster is confirmed in the present work that reports the isolation and characterization of CAL γ , a third lipocalin gene laying just upstream CAL β . Expression studies show an overall pattern of regulation for CAL γ similar to those of Ex-FABP and CAL β , indicating its involvement in similar biological processes. These results suggest the existence of co-regulatory events at the transcriptional level possibly leading to a synergistic action of the three lipocalins during endochondral ossification as well as in inflammation

2. Materials and methods

2.1. Chicken embryos

White Leghorn chicken embryos were incubated at 38°C for various periods of time. Embryos were staged according to Hamburger and Hamilton (1951).

2.2. Cell culture

Cell culture methods have been extensively described elsewhere (Castagnola et al., 1986). Briefly, primary cultures of chondrocytes were isolated from 6-day embryo tibia (stage HH 29–30) by trypsin/collagenase digestion. Dedifferentiated chondrocytes were obtained after 3 weeks of passing the cells in monolayer. To induce re-differentiation, the expanded cells were transferred into suspension culture on dishes coated with 1% agarose and maintained for 3–4 weeks until a homogenous population of single isolated hypertrophic cells was obtained. Culture medium was Coon's modified Ham's F-12, supplemented with 10%

fetal calf serum. When indicated, dedifferentiated cells were stimulated overnight with 10 μ g/ml LPS in Ham's F-12 depleted of serum.

2.3. Genomic and cDNA clones: generation and sequencing

The genomic clone herein analyzed (pGD15) was originally isolated and used to characterize the Ex-FABP promoter and to sequence and isolate CAL β gene (Pagano et al., 2002). Briefly, a chicken genomic phage library in EMBL3 was purchased from Clontech Lab. Inc (Palo Alto, CA, USA). Dilutions of the library were made and the *Escherichia coli* NM358 host strain was grown and infected with standard techniques (Sambrook et al., 1989). Cells were plated and plaquelift hybridization was carried out with the ^{32} P-labeled pDR20 cDNA coding for the Ex-FABP protein (Cancedda et al., 1990). The positive plaque pGD15 was isolated and used to re-infect the host strain for amplification. After growth, the phage DNA was purified and the genomic insert (19 Kb) was subcloned at the *Sal*I site of the plasmid pBluescribe (pBS +/–, Stratagene, La Jolla, CA, USA). The clone pGD15 was sequenced on both strands by the dideoxynucleotide chain termination method (Sanger et al., 1977) using the Thermo Sequenase™ Kit (Amersham Life Science Inc, Little Chalfont, Buckinghamshire, UK).

The BAC genomic clone (bw093F21) positive for CAL γ was identified by PCR-screening of the Wageningen chicken BAC library (Crooijmans et al., 2000). The PCR reaction was performed using the sense 5'-GAACAGTGC-GAGAAGAGGAA-3' primer (nt 1403–1422) and the anti-sense 5'-TAGGATGAGGATCTCCTCGT-3' primer (complementary to nt 1923–1942). Annealing temperature was 56°C and the PCR reaction was carried out for 35 cycles. The gene encoding CAL γ was identified upstream the CAL β promoter. The full sequence of the gene has been deposited to GenBank™ Data Base with the accession number AY082334.

A full-length cDNA clone was generated by RT-PCR using GeneAmp RNA Core Kit (Applied Biosystems, Branchburg, NJ, USA). The template for reverse transcription was total RNA extracted from a 9-day chicken embryo (stage HH 35). The PCR primers, deduced from the genomic sequence, were (see Fig. 1A): 5'-GACATGCAAGC-CACGCTGCT-3' (nt 524–543) and 5'-TAAGAGGGA-CAGGGGCGAG-3' (complementary to nt 2459–2476). Annealing temperature was 64°C and the PCR reaction was carried out for 25 cycles. The PCR product (601 bp) was gel purified, sequenced and cloned at the *Bam*HI/*Hind*III sites of the expression vector pQE30 (Quiagen, Washington, DC, USA).

Gene and cDNA sequences were further computer-analyzed for structure, function and homology determinations (Grail version 3.1, Protein Predict, GeneScan, MegAlign, NetPhos, Ipsort Prediction, Signal PV1.1, Prot Param Tools, 3D pssm and SwissProt Database).

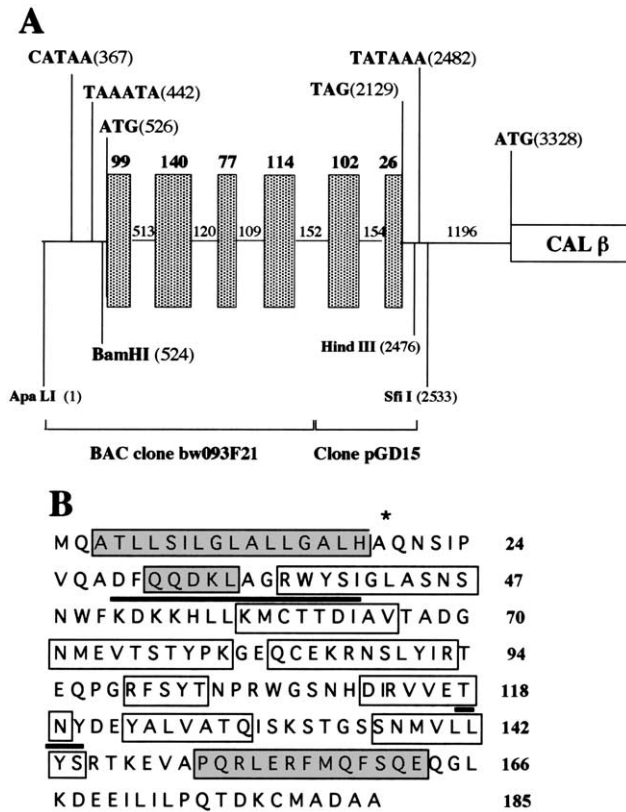


Fig. 1. CAL γ gene and protein. Panel A: Exon/intron structure of CAL γ gene. An Apa LI/Sfi I genomic fragment is analyzed. The portions derived from the genomic clone pGD15 and from the BAC clone bw093F21 are shown at the bottom of the schema. Exons are boxed and the respective exon and intron sizes (in nt) are indicated. Consensus sequences of the minimal promoter (TATA and CAAT boxes), and start and stop codons are indicated. The distance between the CAL γ stop codon (TAG) and the initiation of CAL β open reading frame (ATG) corresponds to 1196 bp. Panel B: CAL γ aminoacid sequence. The two main lipocalin domains are underlined. The eight anti-parallel β -sheets are boxed whereas the α -helices are shown by a gray background. The asterisk denotes the site of cleavage of CAL γ signal peptide. Numbers of residues are indicated on the right.

2.4. Total RNA preparation

RNA was extracted with the guanidinium isothiocyanate procedure of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) from cultured dedifferentiated and re-differentiating chondrocytes, and from tissues harvested from 18-day chicken embryos (stage HH 44) (brain, heart, liver, intestine, muscle, gizzard and sternum).

2.5. Real-time quantitative RT-PCR

Levels of CAL γ mRNA were measured by real-time quantitative RT-PCR using the PE ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Branchburg, NJ, USA). Levels of types I, II and X collagens (accession numbers X02657, L00063 and M13496, respectively) were analyzed in parallel. Measure of the expression

of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, accession number K01458) was also included as endogenous control. The sequences of forward and reverse primers and of the TaqMan[™] fluorogenic probes, as designed by the Primer Express 1.5 software, were:

CAL γ : forward 5'-CTGTCACTGCAGATGGCAACAT-3' (nt 1233–1254)
reverse 5'-CGTGGGTTGGTGTAGCTGAAC-3' (complementary to nt 1456–1476)
probe 5'-FAM-CCTCTTCTCGCACTGTTTACCCTTGG-TAMRA-3'

GAPDH: forward 5'-AAAGTCGGAGTCAACGGATTG-3'
reverse 5'-TGTAACCATGTAGTTCAGATC-GATGA-3'
probe 5'-VIC-CGTATTGGCCGCTGTCACCA-TAMRA-3'

type I coll: forward 5'-GGCTCTGCAACACAAG-GAGTCT-3'
reverse 5'-CCTTCCGCCCTGCAGAT-3'
probe 5'-FAM-CCTCACTCACATATTGGCTTGTTGCTAGG-TAMRA-3'

type II coll: forward 5'-GAGGGCAACAGCAGGTTTAC-3'
reverse 5'-TTCTGCGACCGGTACTCGAT-3'
probe 5'-FAM-CGGCTGCACAAAACACACTGGC-TAMRA-3'

typeX coll: forward 5'-AGGCAGTGCTGTCATTGATCTCATGGA-3'
reverse 5'-TCAGAGGAATAGAGACCATTGGATT-3'
probe 5'-FAM-TCAAGTGTGGCTCCAGCTGCCAAA-TAMRA-3'

All probes were located at the junction between two exons. During PCR amplification, 5' nucleolytic activity of Taq polymerase cleaves the probe, separating the 5' reporter fluorescent dye from the 3' quencher dye. Threshold cycle, Ct, which correlates inversely with the target mRNA levels, was measured 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 constructed from stock cDNA dilutions, and divided by the target quantity of the calibrator according to the manufacturer's instructions.

2.6. In situ hybridization

In situ hybridization was carried on 9 and 13-day embryos (stages HH 35 and 39) as described by Zerega et al. (1999). To probe the sections, a partial cDNA was generated by RT-PCR on total RNA using the primers: 5'-GAA-CAGTGCGAGAAGAGGAA-3' (nt 1403–1422) and 5'-TAGGATGAGGATCTCCTCGT-3' (complementary to nt

1923–1942). The PCR product (279 bp) was subcloned at the *SalI/XbaI* sites of pBluescript KS⁺II (Stratagene, La Jolla, CA, USA). Sense and antisense probes, labeled with digoxigenin-UTP (Roche Molecular Biochemicals, Indianapolis, IN, USA), were synthesized on the linearized plasmid using T7 and T3 RNA polymerases. Hybridization and washing temperature was 52°C, except for the last three washes that were done at 55°C.

3. Results

3.1. CAL γ is a new member of the lipocalin superfamily

We have recently reported that in the chicken genome, Ex-FABP and CAL β genes are organized in tandem (Pagano et al., 2002). Searching for additional member(s)

of a putative cluster, we sequenced the genomic clone pGD15 upstream the CAL β transcription start site. Grail (Vers 3.1) and GeneScan analysis of this region retrieved two exons, their intermediate intron and a 3' untranslated sequence including a polyadenylation signal. These two exons presented structure and size conservations as well as typical lipocalin features that we had already observed for the first two members of the cluster. The upstream intron flanking this initial gene sequence was interrupted by the *SalI* cloning site of the pGD15 insert, indicating that most of the 5' end of the gene was missing on that clone. Thus, we screened a chicken genomic BAC library and isolated one positive colony (bw093F21) whose sequence was combined with that of pGD15 to create the whole gene (Fig. 1A). This gene is 2040 bp long, from TATA box to polyadenylation signal, and is separated from the ATG of the downstream CAL β gene by a non-coding region of 1196 bp. It contains

1	M Q - - - A T L L S I - - - L G L A L L G A L H A Q N S - -	CAL γ
1	M K - - - G L V L S - - - F A L V A L S A L C V Y G D - -	Prostaglandin D2 synthase
1	M - G L G V L - - - - C L A L V L L G V L Q R Q A Q D S	N-GAL
1	M R S L S G L L L L L T A C L A V N A S S V P T L P D D - -	α -1 microglobulin
1	M R T L - - - - - A L S L A L A L L C L L H T E A A - -	Ex-FABP
23	- - - - - I P V Q A D F Q Q D K L A G R W Y S	CAL γ
22	- - - - - V P I Q P D F Q E D K I L G K W Y G	Prostaglandin D2 synthase
24	T Q N L I P A P P L I S V P L Q P G F W T E R F Q G R W F V	N-GAL
29	- - - - - I Q V Q E N F D L S R I Y G K W F N	α -1 microglobulin
22	- - - - - A T V P - - - D R S E V A G K W Y I	Ex-FABP
41	I G L A S N S N W F K D K K H L L K M C T T D I A V T A D G	CAL γ
40	I G L A S N S N W F Q S K K Q Q L K M C T T V I T P T A D G	Prostaglandin D2 synthase
54	V G L A A N A V - Q K E R Q S R F T M Y S T - I Y E L Q E D	N-GAL
47	V A V G S T C P W L K R F K E K M T M S T V V L I A G P T S	α -1 microglobulin
37	V A L A S N T D F F L R E K G K M K M V M A R I S F L G E -	Ex-FABP
71	N - M E V T S T Y P K G E Q C E K R N S L Y I R T E Q P G R	CAL γ
70	N - L D V V A T F P K L D R C E K K S M T Y I K T E Q P G R	Prostaglandin D2 synthase
82	N S Y N V T S I L V R G Q G C R Y W I R T F V P S S R P G Q	N-GAL
77	K E I S V T N T H R R K G V C E S I S G T Y E K T S A D G K	α -1 microglobulin
66	D E L E V S Y A A P S P K G C R K W E T T F K K T S D D G E	Ex-FABP
100	F S Y T N P R W G S N H - - - D I R V V E T N Y D E Y A L V	CAL γ
99	F L S K S P R Y G S D H - - - V I R V V E S N Y D E Y T L M	Prostaglandin D2 synthase
112	F T L G N I H S Y P Q I Q S Y D V Q V A D T D Y D Q F A M V	N-GAL
107	F L Y H K A K W N I T M - - - E S Y V V H T N Y D E Y A I F	α -1 microglobulin
96	V Y Y S E - - - E A E K - - - T V E V L D T D Y K S Y A V I	Ex-FABP
127	A T Q I - S K S T G S S N M V L L Y S R T K E V A P Q R L E	CAL γ
126	H T I K - T K G N E V N T I V S L F G R R K T L S P E L L D	Prostaglandin D2 synthase
142	F F Q K T S E N K Q - Y F K V T L Y G R T K G L S D E L K E	N-GAL
134	L T K K L S R R H G P T I T V K L Y G R E P Q L R E S L L E	α -1 microglobulin
120	F A T R V - K D G R T L H M M R L Y S R S R E V S P T A M A	Ex-FABP
156	R F M Q F S Q E Q G L K D E E I L I L P Q T D K C M A D - -	CAL γ
155	K F Q Q F A K E Q G L T D D N I L I L P Q T D S C M S E - -	Prostaglandin D2 synthase
171	R F V S F A K S L G L K D N N I V F S V P T D Q C I D N	N-GAL
164	E F R E V A L G V G I P E D A I F T M P D R G E C V P G E Q	α -1 microglobulin
149	I F R K L A R E R N Y T D E M V A V L P S Q E E C S V D E -	Ex-FABP

Fig. 2. CAL γ alignment with the four most homologous proteins, prostaglandin D2 synthase, neutrophil gelatinase-associated lipocalin (N-GAL), α 1-microglobulin and Ex-FABP. A grey background indicates the conserved aminoacids where the cysteines and the tryptophans involved in the lipocalin folding are further evidenced by boxes. Numbers of residues are indicated on the left.

six exons and five introns and in such, is structurally similar to those encoding Ex-FABP and CAL β . Cloning and sequencing a full-length cDNA confirmed the exon/intron boundaries and the length of each segment.

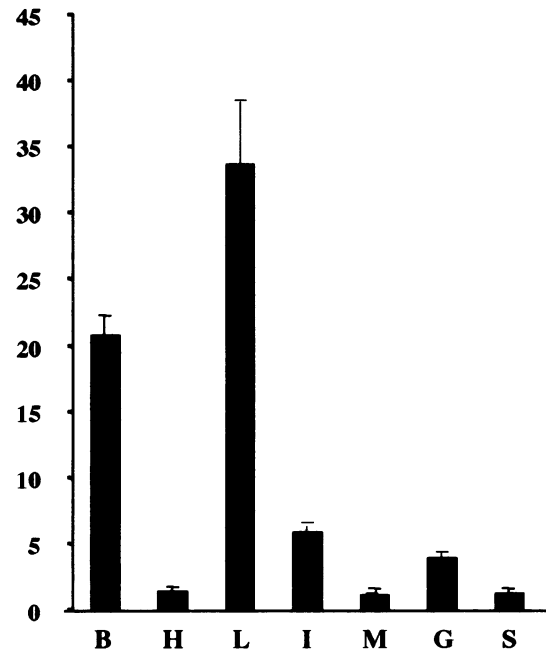
The open reading frame was translated and the resulting peptide sequence was subjected to several computer analyses. A lipocalin domain was identified between the aminoacids (aa) 28 and 41 together with the eight anti-parallel β -sheets and the three α -helices indicative of a β -barrel folding (Fig. 1B) (Flower et al., 2000). This structure was further aligned with a database (3D-pssm) which confirmed that a highly conserved secondary structure is shared by all the lipocalins including the novel one here described. The protein contains 185 aa and a signal peptide most likely cleaved between aa 19 and 20 (LHA-QN) which indicates that it is probably secreted, as previously observed for the two other members of the cluster. Because of the peculiar modulation of its expression in cartilage (see below) and its homology with CAL β , this new lipocalin was named chondrogenesis associated lipocalin γ (CAL γ).

Some physico-chemical parameters of the protein were calculated (Prot Param Tools), like the molecular weight (20,843 Da) and the isoelectric point (6.3). Three serine (at position 101, 109 and 161) and two tyrosine (at position 120 and 123) were indicated as most likely phosphorylated; interestingly, the one at position 120 is always conserved among all the lipocalins.

A BLASTp analysis (non-redundant database) identified *Bufo marinus* prostaglandin D synthase (gi:266472, sp:Q01584) as the protein that shares the highest identity level with CAL γ (57%) (Fig. 2). Lower similarity levels were observed with neutrophil gelatinase-associated lipocalin (31%) and α 1-microglobulin (29%). In the same analysis, identity percentages of 32 and 28 were shared with CAL β and Ex-FABP, respectively.

3.2. CAL γ is prevalently expressed in developing liver and brain

Real-time quantitative RT-PCR experiments were performed to investigate CAL γ gene expression in different chicken tissues. Total RNA preparations from 18 day-embryos were tested for the presence and the relative amount of CAL γ mRNA transcripts (Fig. 3). The lowest level of expression, as referred to the housekeeping gene GAPDH, was observed in the muscle (thus used as calibrator tissue to which all the other values were normalized). The highest value was detected in the liver where CAL γ mRNA transcripts were 33.62-fold more abundant. A high level of transcription was evidenced also in the brain (20.2-fold) while low and comparable signal levels were registered for gizzard, intestine, heart and sternum. The low level of expression of CAL γ in the sternum with respect to tibia (see below) is due to the fact that at variance with long bone cartilage that will eventually undergo endochondral ossification, the sternum remains



Tissue	Fold	S.D.
brain	20,20	1,80
heart	1,33	0,12
liver	33,62	4,73
intestine	5,79	0,47
muscle	1,00	0,16
gizzard	3,86	0,53
sternum	1,10	0,04

Fig. 3. Tissue distribution of CAL γ mRNA revealed by real-time quantitative RT-PCR. Results are presented graphically and numerically. CAL γ mRNA quantities are expressed as an n-fold difference relative to the muscle. The values are means ($\pm$ standard deviation, SD) of three independent determinations. For reference, GAPDH gene expression was measured from the same RNA samples. B, brain; H, heart; L, liver; I, intestine; M, skeletal muscle; G, gizzard; and S, sternum.

essentially permanent immature cartilage throughout life and partially calcify only in the adult; moreover, at the developmental stage we have analyzed, it contains mostly quiescent, resting chondrocytes and a very limited amount of maturing chondrocytes (D'Angelo and Pacifici, 1997).

3.3. CAL γ expression is modulated during chondrogenesis in vitro and in vivo

Based on the modulation of gene expression we have previously observed for the two other members of the lipocalin cluster during chondrogenesis (Pagano et al., 2002), we first investigated CAL γ transcription profile during chondrocyte differentiation in vitro using real-time quantitative RT-PCR. The levels of expression of types I, II and X collagens were assessed in parallel to possibly correlate CAL γ with various stages of chondrocyte maturation.

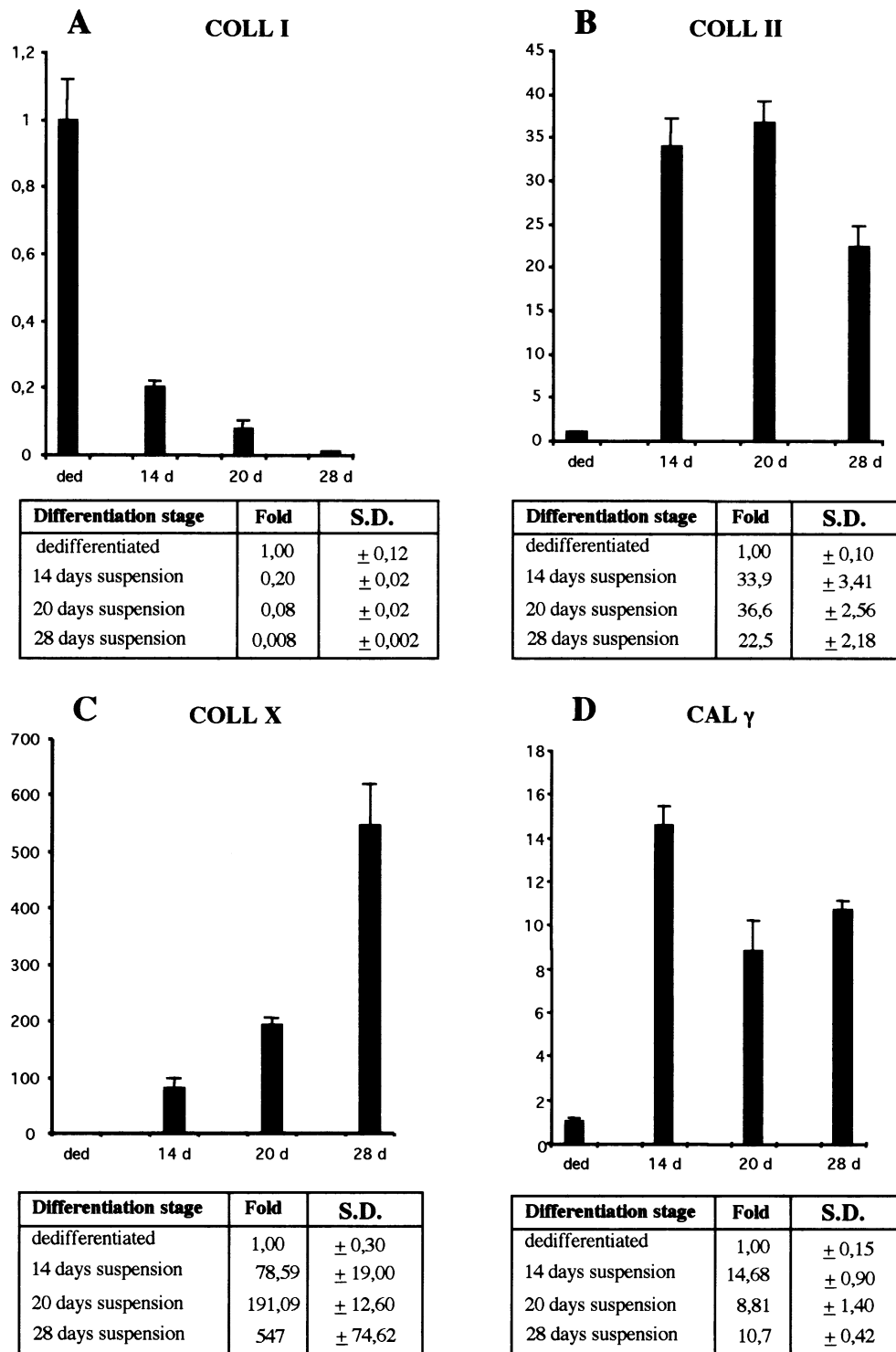


Fig. 4. Comparative expression of CAL γ and types I, II and X collagen mRNAs during chondrogenesis in vitro. Real-time quantitative RT-PCR was performed on RNA derived from dedifferentiated cells maintained in monolayer and differentiating chondrocytes grown in suspension (days 14 and 20: maturing pre-hypertrophic cells; day 28: hypertrophic chondrocytes). For each gene, the value obtained in differentiating chondrocytes is expressed as an n-fold difference relative to the dedifferentiated cells. Results are means ($\pm$ standard deviation, SD) of three independent determinations. For reference, GAPDH gene expression was measured from the same RNA samples. Panel A refers to type I collagen expression, panel B to type II collagen, panel C to type X collagen and panel D to CAL γ .

Samples of mRNA were obtained from embryo tibia chondrocytes cultured either as cells dedifferentiated in monolayer or as cells differentiating in suspension. GAPDH was used as reference housekeeping gene to which expression levels of CAL γ and of the various collagens were reported.

As we previously demonstrated (Castagnola et al., 1986, 1988; Quarto et al., 1993), dedifferentiated cells synthesized high amounts of type I collagen (Fig. 4A), but essentially no type II (Fig. 4B) and type X (Fig. 4C) collagens. At this immature stage, also CAL γ was expressed at a very low level (Fig. 4D). When transferred into suspension culture, those dedifferentiated cells re-entered the chondrogenic pathway. In this process, the differentiating cells gradually ceased synthesizing type I collagen. Maturing pre-hypertrophic cells expressed high levels of type II collagen

that slightly decreased at the hypertrophic stage. The level of type X collagen progressively increased during maturation reaching a maximal value in hypertrophic cells. The expression profile of CAL γ resembles that of type II collagen as the amounts of mRNA were high in pre-hypertrophic and hypertrophic chondrocytes. Thus, this novel lipocalin can be defined as a developmentally regulated protein.

To verify the expression profile of CAL γ in vivo, total RNA was also extracted from tibia cartilage of 10 and 18-day-old embryos (stages HH 36 and 44, respectively). Real-time quantitative RT-PCR analysis revealed that CAL γ mRNA was increasingly expressed according to the developmental stage, being seven times more abundant at the later stage (not shown). To identify the area(s) of expression in the developing bone, in situ hybridizations were performed on embryo tibia sections challenged with a

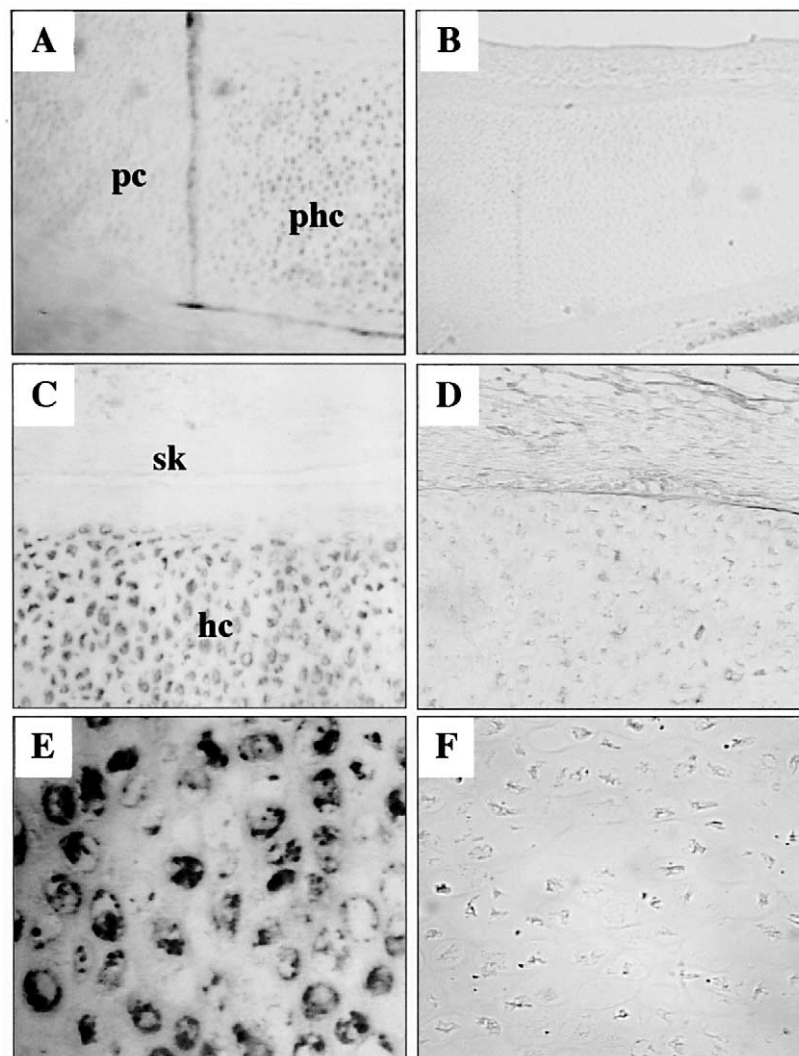


Fig. 5. CAL γ expression in developing chicken tibia (stage HH 35). Panels A–F: mRNA detection by in situ hybridization in the tibia area of the bone-forming cartilage. Panels A, C and E show the hybridization obtained with the anti-sense probe. Panels B, D and F are the corresponding negative controls obtained with the sense probe. Magnification: panels A and B, 10 $\times$; panels C and D, 20 $\times$; panels E and F, 40 $\times$. sk: skeletal muscle, pc: proliferating chondrocytes; phc: pre-hypertrophic chondrocytes; hc: hypertrophic cartilage.

CAL γ antisense probe. At 9 days of development (stage HH 35), a strong hybridization signal was observed in the region of hypertrophic chondrocytes (Figs. 5C,E). In agreement with the RT-PCR results reported above on embryonic tissues, the surrounding skeletal muscle appeared essentially negative (Fig. 5C). A very weak signal was detected in the proliferating zone adjacent to the pre-hypertrophic one where a significant positivity could also be observed (Fig. 5A). Serial sections hybridized with the sense probe resulted negative (Figs. 5B,D,F). Similar results were obtained on tibia sections of 13-day-old embryo (stage HH 39) (not shown).

3.4. CAL γ is induced by an inflammatory agent

The previous finding that both Ex-FABP (Cermelli et al., 2000) and CAL β (Pagano et al., 2002) are strongly induced by inflammatory agents prompted us to verify whether CAL γ presents a similar responsiveness. Dedifferentiated

chondrocytes were stimulated with the endotoxin LPS and real-time quantitative RT-PCR analysis was performed on total RNA preparations before and after stimulation. As seen in Fig. 6, LPS treatment significantly stimulated CAL γ mRNA expression that showed a 70-fold increase in treated cells as compared to the untreated counterpart. By contrast, type X collagen mRNA level was not affected by the inflammatory agent, ruling out an overall effect of LPS on cellular biosynthesis and/or differentiation.

4. Discussion

Here we report the isolation and characterization of a novel chicken lipocalin. In view of the expression data we have obtained, the protein is the third member of the superfamily to be strongly modulated during chondrocyte differentiation and is thus referred to as CAL γ . The gene encoding CAL γ has been identified upstream the promoter of the CAL β gene. CAL β and Ex-FABP genes are themselves organized in sequence (Pagano et al., 2002). Thus at the chromosomal level, the three genes form a genomic cluster that might include additional, yet unidentified, members. All three genes present the same structural organization, being composed of six exons and five introns. Moreover, the size distribution of these exons and introns is quite similar in all genes. This observation provides a new element supporting the hypothesis of a tandem repeated duplication of an ancestral lipocalin gene at the origin of the cluster, as it has been already suggested for mammalian clusters (Salier, 2000). According to the comparative sequence analysis, prostaglandin D synthase displays the highest homology with all three proteins and may be the candidate ancestor.

The conserved secondary structure motifs of CAL γ are typical diagnostic elements of the lipocalin family as they determine the β -barrel folding built around the eight β -sheets (Flower et al., 2000). Also the lipocalin domain found at position 28–41 and considered, in most of the members of the protein family, as part of the binding site for a hydrophobic molecule, clearly demonstrates the lipocalin nature of this protein.

We also investigated some functional aspects of CAL γ to get insight into its possible physiological role. Firstly, a quantitative tissue distribution analysis indicated the liver and the brain as the main sites of expression. The liver is the principal source of acute phase response proteins, including some lipocalins. The high level of CAL γ that we found in this tissue supports the hypothesis of its possible involvement in inflammatory responses, as also indicated by the experiments of stimulation with LPS that we have performed. The significant expression of CAL γ mRNA in the brain is in turn suggestive of a specific action of the protein in that tissue, similarly to the preferential tissue distribution and the involvement in specific biological pathways already described for prostaglandin D2 synthase

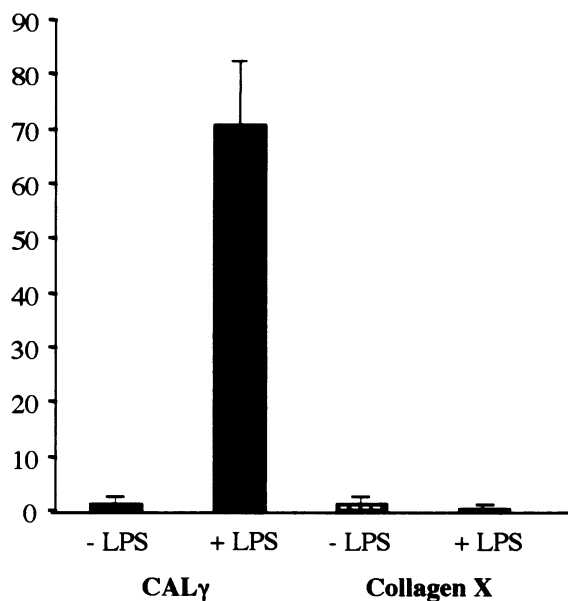


Fig. 6. Induction of CAL γ by inflammatory agents. Comparative expression of CAL γ and type X collagen mRNAs upon stimulation with LPS. Real-time quantitative RT-PCR was performed on RNA derived from treated and untreated cells. For each gene, the value obtained after LPS stimulation is expressed as an n-fold difference relative to the untreated cells. Results are means ($\pm$ standard deviation, SD) of three independent determinations. For reference, GAPDH gene expression was measured from the same RNA samples.

in human and mouse central nervous system (Beuckmann et al., 2000; Urade and Hayaishi, 2000). Low but significant levels of CAL γ expression were observed in all the other tissues examined. Such a ubiquitous distribution had been previously observed by Northern blot analysis for Ex-FABP (Dozin et al., 1992) and by semi-quantitative RT-PCR for CAL β (Pagano et al., 2002).

Our data further show that CAL γ expression correlates with two important biological processes where also CAL β and Ex-FABP are modulated (Pagano et al., 2002), the endochondral bone formation and the inflammatory response. As for these two lipocalins, CAL γ mRNA is increasingly synthesized during chondrocyte differentiation both in vivo and in vitro, and highly accumulates upon LPS stimulation. The similarity in genomic localization, ubiquitous tissue distribution, expression profile during chondrogenesis and in response to inflammatory agents indicate that the genes encoding CAL γ , Ex-FABP and CAL β are most probably co-regulated at the transcriptional level and that the three proteins may share a related function.

A high concentration of proteases within the pericellular zone is common to both remodeling cartilage and inflamed tissue. One may thus tentatively propose the existence of a direct interaction between secreted lipocalins and proteases surrounding the cell, possibly leading to an inhibitory effect on the proteolytic activity. This mechanism would protect the cell from degradation and subsequent death. Two hybrid experiments in yeast are currently in progress to validate the hypothesis that such a physical molecular interaction is indeed occurring.

Acknowledgements

This work was partially supported by funds from the Associazione Italiana per la Ricerca sul Cancro (AIRC, Italy) and MURST.

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