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The Developmentally Regulated Avian Ch21 Lipocalin Is an Extracellular Fatty Acid-binding Protein*

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Ch21, a developmentally regulated extracellular protein expressed in chick embryos and in cultured chondrocytes, was expressed in the baculovirus system, and the recombinant protein was purified to homogeneity by gel-filtration chromatography. Separation of two isoforms was achieved on an ion-exchange column. Previous work had shown that Ch21 belongs to the superfamily of lipocalins, which are transport proteins for small hydrophobic molecules. Studies were performed to identify the Ch21 ligand.

By analysis of recombinant Ch21 on native polyacrylamide gel electrophoresis and by Lipidex assay, the binding of fatty acid to the protein was shown and a preferential binding of long-chain unsaturated fatty acids was observed. Both isoforms had the same behavior. The binding was saturable. Stoichiometry was about 0.7 mol of ligand/mol of protein. The protein binds the ligand in its monomeric form. Calculated dissociation constants were 2×10^{-7} M for unsaturated fatty acids and 5×10^{-7} M for stearic acid. The binding was specific; other hydrophobic molecules, as retinoic acid, progesterone, prostaglandins, and long-chain alcohols and aldehydes did not bind to the protein. Short-chain fatty acids did not bind to the protein.

Ch21, also present in chicken serum, represents the first extracellular protein able to selectively bind and transport fatty acid in extracellular fluids and serum. We propose to rename the Ch21 protein as extracellular fatty acid-binding protein (Ex-FABP).

In the last several years, our laboratory has focused on the cellular and molecular mechanisms that control endochondral bone formation. The process is characterized by the differentiation of prechondrogenic mesenchymal cells to resting, proliferating, and hypertrophic chondrocytes (1–8) and then by the replacement of hypertrophic cartilage by bone, *i.e.* cartilage calcification, erosion, invasion by blood vessels, and onset of osteogenesis. Hypertrophic chondrocytes play a critical role in the last part of the process. In fact they can undergo further differentiation to osteoblast-like cells both *in vitro* (9) and, in selected bone regions, *in vivo* (10–14) and are essential to the deposition of the first bone on newly synthesized bone sialoprotein-rich matrix ("bone priming").[1] Recent data suggest that the initial bone deposition may be activated through the expression of the *Krox-20* gene by hypertrophic chondrocytes (15). In addition, hypertrophic chondrocytes express and secrete, into the extracellular matrix, diffusible factors promoting angiogenesis (16). During the whole process, it is possible to observe the progressive synthesis of several proteins whose temporal and spatial appearance marks each cell differentiation stage. Hypertrophic chondrocytes secrete relatively large amounts of transferrin both *in vitro* and *in vivo* (17, 18). The receptors for this factor are highly expressed in surrounding osteoblasts and preosteoblasts, suggesting a paracrine stimulation of these cells.

Hypertrophic chondrocytes synthesize and secrete large amounts of Ch21 protein, a low molecular weight protein belonging to the lipocalin superfamily (19–22). Members of this family have a molecular weight of about 20,000, a 20–30% homology, and share a basic framework for binding and transport of small hydrophobic molecules (23). The specific ligands for some of these proteins have been identified. They represent a wide variety of hydrophobic molecules: retinol (retinol-binding protein, Ref. 24; purpurin, Ref. 25; β-lactoglobulin, Ref. 23), retinoic acid (epididymal retinoic acid-binding protein, Ref. 26), biliverdin (insecticyanin, Refs. 27 and 28), odorants (odorant-binding protein, Ref. 29), progesterone (apolipoprotein D, Ref. 30), pheromone (urinary proteins, Ref. 31). In the present study, we have investigated the nature of the ligand for the Ch21 protein and its possible function. We present evidence that the recombinant Ch21 expressed in baculovirus-infected cells selectively binds long chain fatty acids. Unsaturated fatty acids, like oleic, linoleic, and arachidonic acid, are preferentially bound. The unsaturated fatty acids bind to the monomeric form of the protein with a K_d in the order of 10^{-7} M. The presence of the Ch21 protein in the bone growth plate and its synthesis by hypertrophic chondrocytes and osteoblasts raise the question of the importance of lipid and fatty acid metabolism in cartilage development and bone formation.

Interestingly Ch21 is also detectable in serum, in agreement with the idea that its role may be more general.

Intracellular fatty acid-binding proteins have been purified from a variety of tissues, including intestine, liver, heart, mammary, Schwann cell, and adipose (32–37). These intracellullar-binding proteins have a molecular weight of about 14,000. Extracellular fatty acids are transported by albumin in blood stream (38). To our knowledge, the Ch21 represents the first

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[1] M. Riminucci, C. Gentili, J. N. Bradbeer, F. Descalzi, R. Cancedda, and P. Bianco, submitted for publication.

example of an extracellular protein able to selectively bind and transport fatty acids in extracellular fluids and blood.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]Linoleic acid, [1-^{14}C]oleic acid, [1-^{14}C]arachidonic acid, [1-^{14}C]stearic acid (50–60 mCi/mmol) were from Amersham International Srl., Buckinghamshire, United Kingdom; [U-^{14}C]linoleic acid (1 Ci/mmol) and [1-^{14}C]myristic acid (50–60 mCi/mmol) were purchased from NEN Du Pont De Nemours, Dreiech, Germany. Nonradioactive fatty acids, oleyl alcohol, cis-13-octadecenale, hydroxyalkoxypropyl dextran type III, prostaglandin E_2, and prostaglandin D_2 were purchased from Sigma.

Centricon 10 and Centriprep 10 were purchased from Amicon, Beverly, MA. Polyacrylamide gel electrophoresis reagents were purchased from Bio-Rad. Ethyl acetate was HPLC grade from BDH, Poole, United Kingdom. Mono Q and Superdex 75 columns were purchased from Pharmacia Biotech, Uppsala, Sweden. ACA 54 resin was purchased from IBF Biotecnics, Villeneuve la Garenne, France. SF900 medium was from purchased Life Technologies, Inc., S. Giuliano Milanese, Italy.

Expression of Recombinant Ch21 in Baculovirus-infected Cells

Ch21 protein was produced in the baculovirus system according to published procedure (39). The PST1-ECORI pDr5 fragment (766 base pairs) of the pDr5 cDNA coding for the Ch21 (22) was inserted in the *Bam*HI site of pAcYM1. Recombinant baculoviruses were obtained by cotransfection of Sf9 cells with wild type AcMNPV and pACyMI/pDr5 and purified by limiting dilution and dot hybridization.

For production of Ch21 protein, Sf9 cells were grown in SF900 medium without fetal calf serum and infected with purified recombinant virus at 5 plaque-forming units/cell. At 3 days post-infection, the culture medium was recovered. After centrifugation, it was analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the gel was stained with silver.

In a typical infection, 250 ml of medium were collected from 5–6 × 10^8 cells.

Chondrocyte Culture

Cell culture was performed according to procedures described previously (19).

Ch21 Protein Purification

The Ch21 protein was purified from the spent culture medium of hypertrophic chondrocytes and from the culture medium of baculovirus-infected cells by the same procedure. Aliquots of media from baculovirus-infected cells were concentrated about 10 times with a Centriprep 10 to a volume of 5 ml, dialyzed against 30 mM Tris-HCl, pH 7.4, 0.3 M NaCl, and loaded on a 2.5 × 35-cm ACA54 column equilibrated with the same buffer. The column was developed at 20 ml/h, and fractions of 2 ml were collected. The culture media from baculovirus-infected cells yielded a substantially pure preparation of Ch21 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis after this purification step. Media from cultured chondrocytes were concentrated 100 times by ammonium sulfate precipitation, followed by Centriprep 10 filtration. The Ch21 purified from chondrocyte culture medium required an additional purification step on a Mono Q column (see next paragraphs).

Separation of Ch21 Isoforms

In the medium from both baculovirus-infected cells and chondrocytes, the Ch21 protein is present in two isoforms (~50% each) that differ in the presence of two additional alanines at the amino terminus (40). Separation of the two isoforms was achieved by HPLC gradient chromatography on a Mono Q column from Pharmacia: buffer A (20 mM phosphate buffer, pH 8); buffer B (20 mM phosphate buffer, pH 8, 0.25 M NaCl); gradient, 100% B in 60 min; flow rate, 0.5 ml/min.

Polyacrylamide Gel Electrophoresis

Electrophoresis of Ch21 Protein in Native Conditions—Electrophoresis was performed essentially as described (41). The lower gel was 10% acrylamide in 0.37 M Tris-HCl, pH 9.2. The upper gel was 4.5% acrylamide in 44 mM Tris phosphate, pH 6. The upper chamber running buffer was 46 mM Tris glycine, pH 8.4. The lower chamber running buffer was 63 mM Tris-HCl, pH 7.4.

Ch21 protein was incubated in phosphate-buffered saline at different concentrations of fatty acids at 4 °C overnight. Fatty acids were dissolved in ethanol and added to the reaction mixture in a volume that did not exceed 1% of ethanol. After the incubation, 20% glycerol and 0.005% bromphenol blue were added and the sample was loaded onto the gel. Electrophoresis was performed at 20 mA at 4 °C.

When indicated, extraction of the endogenous ligand was performed before electrophoresis. The protein was acidified to pH 4 with acetic acid and extracted twice with 12 volumes of ethyl acetate for 45 min at 4 °C. The remaining ethyl acetate was removed from the aqueous phase by evaporation during a brief centrifugation under vacuum.

After electrophoresis, the proteins were detected by silver staining. When proteins preincubated with radioactive fatty acids were analyzed, an aliquot of nonradioactive control protein was run in parallel. After electrophoresis, the gel was divided into two parts. The half with the non-radioactive control protein was detected by silver stain, and the half with the radioactive sample was exposed for autoradiography.

Electrophoresis of Proteins in Sodium Dodecyl Sulfate Denaturing Conditions—Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to published procedures both in reducing and unreducing conditions (42). Gel concentration was 15%.

Radiolabeled Fatty Acid Binding Assay

Binding assay was performed essentially as described (43). Hydroxyalkoxypropyl dextran type III from Sigma (equivalent to Lipidex 1000 from Packard Instruments) was utilized. Before the assay, the resin was swollen in ethanol and extensively washed with PBS[2] by filtration/centrifugation in a small column. The Ch21 protein was incubated with the radioactive ligand (dissolved in ethanol; final concentration of ethanol 1% or less) overnight at 4 °C in 250 μl of PBS in siliconized Eppendorf tubes. After incubation, 10 mg of the equilibrated lipophilic resin were added, the mixture was shaken and the incubation was continued for 10 min at 0 °C. After centrifugation in an Eppendorf centrifuge for 3 min at 4 °C, 200 μl of supernatant were carefully withdrawn and counted for radioactivity.

Determination of Protein M_r on Superdex 75 Column

A 10 mm × 30-cm Superdex 75 FPLC column (Pharmacia) was calibrated with molecular weight markers: bovine serum albumin, ovalbumin, soybean trypsin inhibitor, and cytochrome *c* (all proteins from Sigma) in PBS. The Ch21 protein (6 μM) was first incubated with ^{14}C-labeled linoleic acid (6 μM) in 0.5 ml of PBS overnight at 4 °C and then analyzed on the Superdex 75 column. A control of unlabeled Ch21 protein was run immediately after the molecular weight determination of the labeled protein. Flow rate was 0.5 ml/min. A fraction was collected every min, and 100 μl of each fraction were counted for radioactivity. Radioactive fractions were concentrated in Centricon 10 and analyzed on native polyacrylamide gel electrophoresis.

Western Blotting

After polyacrylamide gel electrophoresis, the gel was blotted to a BA85 nitrocellulose membrane (Schleicher & Schüll GmbH, Dassel, Germany) according to the procedure described by Towbin *et al.* (44). The blot was saturated for 16 h with 5% nonfat cow milk in TTBS buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20), washed several times with TTBS and incubated with purified anti Ch21 antibodies in 5% low fat milk in TTBS for 3 h at room temperature. After washing with TTBS, the protein was detected with an alkaline phosphatase-conjugated anti-rabbit IgG (Boehringer Mannheim GmbH, Mannheim, Germany (1:2,000 in 5% low fat milk in TTBS). After additional washing with TTBS, the blot was washed in a solution of 20 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5. Finally the blot was exposed to the substrate solution (Western Blue stabilized substrate for alkaline phosphatase, Promega, Madison, WI). The reaction was quenched with 20 mM Tris-HCl, 20 mM EDTA, pH 7.2.

RESULTS

Expression of Recombinant Ch21 by Baculovirus-infected Cells

An electrophoretic pattern of proteins released by control and Ch21 recombinant baculovirus-infected Sf9 cells is presented in Fig. 1. A band that was absent in the supernatant of Sf9 cells, infected both with the wild type and a control unrelated recombinant virus, was present in the culture medium of cells infected with the Ch21 recombinant virus. This band had the same electrophoretic mobility of labeled Ch21 from chicken

[2] The abbreviations used are: PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography.

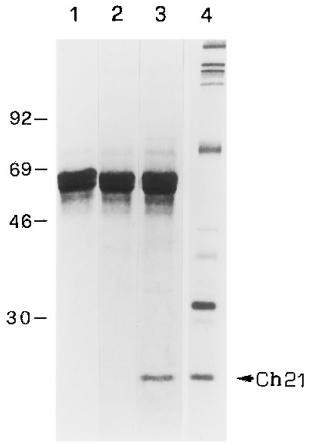

FIG. 1. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins secreted by baculovirus-infected Sf9 cells.** *Lane 1*, control uninfected cells; *lane 2*, control cells infected with an unrelated recombinant virus; *lane 3*, cells infected with the Ch21 recombinant virus; *lane 4*, labeled proteins released by radioactively labeled cultured chicken hypertrophic chondrocytes. After electrophoresis, proteins were detected by silver stain (*lanes 1–3*) or by autoradiography (*lane 4*).

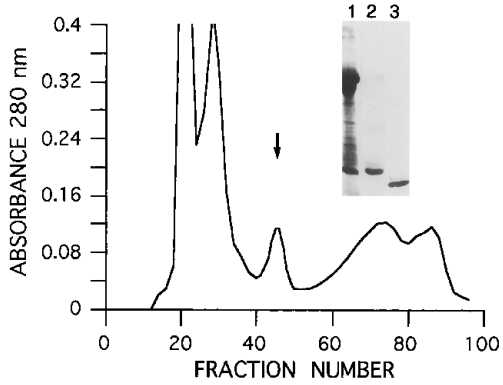

FIG. 2. **Chromatography of proteins from medium of Sf9 cells on ACA54 column.** The *arrow* indicates the peak of elution of Ch21. The insert shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of proteins in the sample applied to the column (*lane 1*) and recovered in the peak (*lanes 2* and *3*). *Lanes 1* and *2* were analyzed under reducing conditions. *Lane 3* was analyzed under non-reducing conditions. The gel was silver-stained.

chondrocytes and was recognized by antibodies raised against the protein purified from chondrocyte medium (see also Fig. 8).

Purification of the Protein and Separation of the Two Isoforms

The Ch21 protein was purified from baculovirus-infected cell medium by gel filtration on ACA 54 column (Fig. 2). The Ch21 recovered from the symmetrical peak in the protein profile appeared substantially pure by polyacrylamide gel electrophoresis both under reducing and non-reducing conditions (Fig. 2, *inset*). In a typical purification, 2–5 mg of Ch21 were obtained from 1 liter of medium.

We have reported the existence in chondrocyte culture medium of two isoforms present in about 50% ratio and differing for the presence of two extra residues of alanine at the amino terminus of one form (40). The two isoforms were also separated by anion exchange chromatography from the purified recombinant protein (Fig. 3). Mass spectra of the two isoforms were acquired using a Hewlett Packard 5989A single quadrupole mass spectrometer equipped with an electrospray ionization source. The obtained mass of the two isoforms (respectively 18,051 and 17,909) was in agreement with the molecular weight calculated from the amino acid sequence of the isoforms purified from chondrocyte medium. Therefore, an identical proteolytic processing of the Ch21 occurs in avian and insect cells.

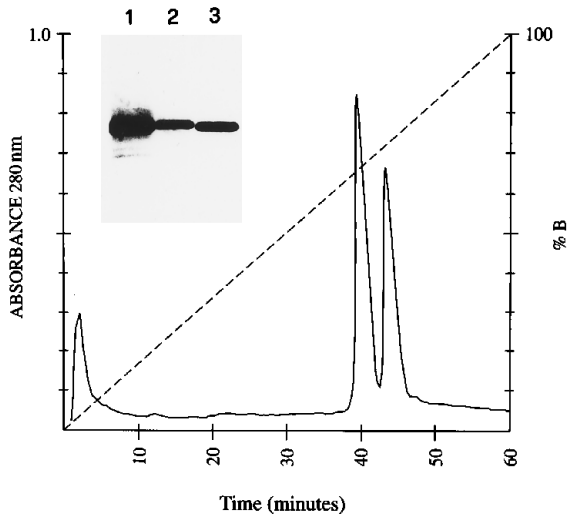

FIG. 3. **Separation of the recombinant Ch21 isoforms on a Mono Q column by HPLC.** The *inset* shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of proteins in the sample applied to the column (*lane 1*) and and of the two isoforms (*lanes 2* and *3*). The gel was run in non-reducing conditions and silver-stained.

Identification of the Ch21 Ligand

Binding of Fatty Acids to the Protein—The purified recombinant Ch21 was analyzed by gel electrophoresis under native conditions following the protocol used to analyze the retinol-binding protein (41). Two forms of the protein, presenting different electrophoretic mobilities due to different electric charges, were separated (Fig. 4). Treatment of the protein with ethyl acetate in acid conditions (pH 4) prior to the electrophoresis resulted in the disappearance of the more anodic form and in the increase in the amount of the less anodic form (Fig. 4*A*). Identical results were obtained with the Ch21 purified from chondrocyte culture medium (not shown). These results indicate that a hydrophobic acid ligand extractable with ethyl acetate is bound to the more anodic form of the protein.

In the attempt to identify the ligand, the purified recombinant protein was incubated with different hydrophobic ligands at different concentrations: retinol, retinoic acid, steroids and fatty acids. We observed a shift in the electrophoretic mobility only with fatty acids (linoleic, oleic, and stearic acid) at concentrations of 10^{-5} M (Fig. 4*B*). The band of the less anodic form was almost completely replaced by a band comigrating with the more anodic form of the purified protein.

Next we incubated the protein with radiolabeled linoleic acid in the absence and in the presence of a 100-fold excess of non-radioactive linoleic acid. After electrophoresis of the incubated samples, the polyacrylamide gel was divided and either stained with silver or dried and exposed for autoradiography. The radioactive ligand binds only to the more anodic form of the protein and is displaced by the non-radioactive ligand excess (Fig. 4*C*).

To investigate whether both isoforms, differing only in alanine residues, were capable of binding fatty acids, we performed experiments with the two isoforms separated by Mono Q column chromatography. After chromatography, both isoforms loose endogenous ligand, although a small amount of ligand-protein complex was still detectable in several protein preparations. Treatment of both isoforms with 10^{-5} M linoleic or oleic acid resulted in the reappearance or in a dramatic increase of the more anodic form (not shown).

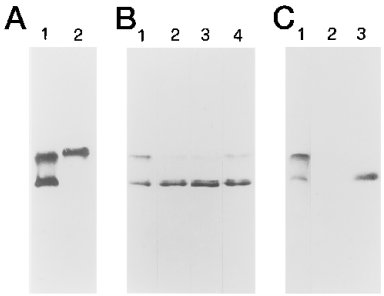

FIG. 4. **Association of fatty acids with the purified recombinant Ch21 analyzed by polyacrylamide gel electrophoresis under native conditions.** *Panel A*, protein (5 μg) before (*lane 1*) and after (*lane 2*) extraction with ethyl acetate. *Panel B*, *lane 1*, control; *lanes 2–4*, Ch21 (3×10^{-6} M) incubated in phosphate-buffered saline (50 μl) with oleic (*lane 2*), linoleic (*lane 3*), and stearic acid (*lane 4*) at 1×10^{-5} M concentration (see "Experimental Procedures"). *Panel C*, Ch21 (6×10^{-6} M) was incubated in phosphate-buffered saline (80 μl) with [^{14}C]linoleic acid 2×10^{-5} M (see "Experimental Procedures"). *Lane 1*, control protein, silver stain. *Lanes 2* and *3*, autoradiography of the gel loaded with the protein incubated with radioactive linoleic acid in the presence (*lane 2*) and in the absence (*lane 3*) of 100-fold excess of non-radioactive linoleic acid.

Dissociation Constant Determination of the Fatty Acid-Ch21 Complex—In preliminary experiments, we determined limiting conditions for Ch21-fatty acid binding. Based on these results, several radioactive fatty acids at 1×10^{-7} M were incubated with the recombinant purified Ch21 at concentrations ranging from 1×10^{-8} to 2×10^{-6} M. Concentrations of Ch21 protein were determined based on amino acid analysis performed in triplicate. Both isoforms of the protein were analyzed in independent assays with identical results. To avoid duplication, only results obtained with one isoform are presented. The binding curves of the protein with linoleic, oleic, arachidonic, stearic, and myristic acid are presented in Fig. 5. Unsaturated fatty acids bind with a K_d in the order of 2×10^{-7} M, while stearic acid binds with a K_d of 5×10^{-7} M and myristic acid with a K_d of 1×10^{-6} M. Calculation of the equilibrium binding constants was performed with the following equilibrium equation from the midpoints of the curves (45).

$$[\text{Ch21-FA}] = \frac{[\text{Ch21}]_0 \times K_a([\text{FA}]_0 - [\text{Ch21-FA}])}{1 + K_a([\text{FA}]_0 - [\text{Ch21-FA}])} \qquad \text{(Eq. 1)}$$

$[\text{Ch21}]_0$ and $[\text{FA}]_0$ are the total concentrations of the protein and the fatty acid, and [Ch21-FA] is the concentration of the complex. K_a is the association constant; K_d is $1/K_a$.

The fatty acid binding to the protein appears specific. Displacement of the radioactive fatty acid by high concentrations of the non-radioactive ligand was complete (data not shown). Other hydrophobic small molecules, such as retinoic acid and progesterone, did not bind to the protein when tested at the same concentration range.

When the binding of fatty acid of different chain lengths, saturated and unsaturated, was tested by a competition assay with labeled oleic acid, we observed that short-chain fatty acid did not displace the binding of oleic acid to Ch21 protein and that medium-chain unsaturated fatty acid displaced the binding better than the saturated fatty acid of the same length (Table I). Interestingly, long-chain aldehyde and alcohol did not displace the oleic acid binding.

Since arachidonic acid was bound with high affinity, we investigated the possible binding of some of its metabolites, namely prostaglandins (Table I). The radioactive arachidonic acid bound to the protein was displaced, as expected, by non-radioactive arachidonic acid. In contrast, the radioactive arachidonic was not displaced by prostaglandin D_2 or prostaglandin E_2.

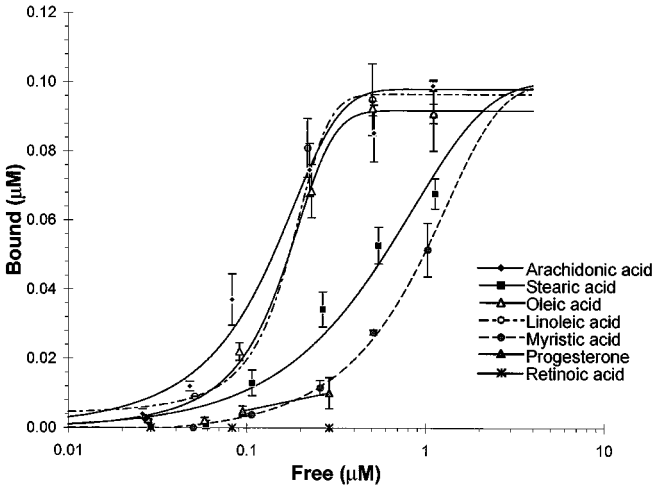

FIG. 5. **Representative binding experiments.** A fixed limiting amount of radioactive hydrophobic ligand (1×10^{-7} M) was incubated with increasing concentrations of recombinant purified Ch21. Each point was assayed in duplicate. Determinations were repeated at least twice. Calculated K_d are: linoleic acid, 1.7×10^{-7} M; oleic acid, 1.7×10^{-7} M; arachidonic acid, 1.6×10^{-7} M; stearic acid, 5.9×10^{-7} M; myristic acid, 9.8×10^{-7} M.

TABLE I
Displacement of binding of radioactive fatty acids with non-radioactive fatty acids and related compounds

Ch21 concentration was 0.24 μM, radioactive fatty acid concentration was 2 μM, non-radioactive competitor concentration was 20 μM. Data are indicated as mean ± standard deviation.

Radioactive fatty acids	Non-radioactive competitor	Radioactive fatty acid bound	Relative binding
		μM	%
Arachidonic acid		0.150 ± 0.012	100
Arachidonic acid	Arachidonic acid	0.010 ± 0.004	6.6
Arachidonic acid	PGE_2	0.160 ± 0.002	106.6
Arachidonic acid	PGD_2	0.180 ± 0.007	120.0
Oleic acid		0.158 ± 0.018	100
Oleic acid	Oleic acid	0.014 ± 0.009	8.7
Oleic acid	Oleyl alcohol	0.150 ± 0.027	94.9
Oleic acid	*cis*-13-Octadecenoic acid	0.047 ± 0.006	29.7
Oleic acid	*cis*-13-Octadecenale	0.150 ± 0.027	94.9
Oleic acid	Palmitic acid	0.108 ± 0.011	68.3
Oleic acid	Palmitoleic acid	0.053 ± 0.005	33.5
Oleic acid	Myristoleic acid	0.057 ± 0.010	36.0
Oleic acid	Caproic acid	0.167 ± 0.006	105.6
Oleic acid	Butyric acid	0.155 ± 0.046	98.1

Molecular Weight of the Fatty Acid-Ch21 Complex—The data of Table I indicate that saturation of binding was obtained with a ligand/protein molar ratio of 0.66:1. Saturation of binding at the same molar ratio was observed when progressively increasing concentrations of arachidonic acid were added to a fixed concentration of protein (Fig. 6). Among the possible explanations for a binding saturation at a molar ratio lower than 1 could be the presence of an inactive fraction of protein, binding of some endogenous ligand or a requirement for a protein dimer for generation of a ligand binding site. To distinguish among these possibilities, we determined the molecular weight of the protein previously incubated with linoleic acid at a saturating concentration (1:1 molar ratio) (Fig. 7). The elution volume of the recombinant Ch21 after chromatography on a Superdex 75 column before and after incubation with the linoleic acid remained the same. In both cases the calculated molecular weight was 18,000, in agreement with the molecular weight determined from the amino acid composition, mass spectrometry, and the sodium dodecyl sulfate-polyacrylamide gel elec-

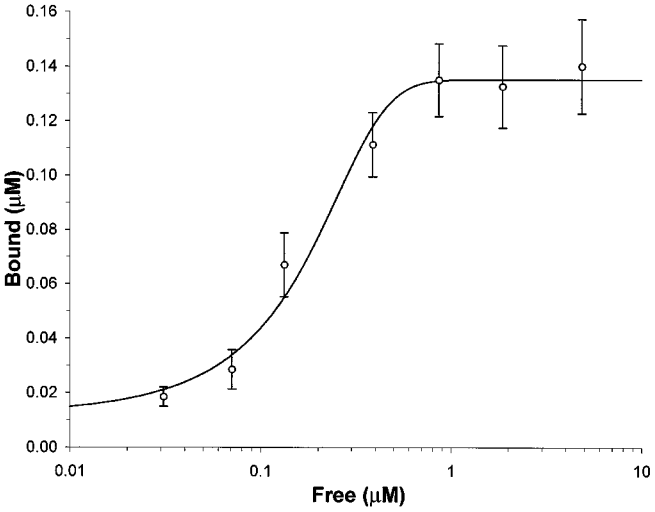

FIG. 6. **Representative saturation experiment.** A fixed limiting amount of radioactive recombinant purified Ch21 (2.4×10^{-7} M) was incubated with increasing concentrations of arachidonic acid (μM). Each point was assayed in duplicate and repeated twice.

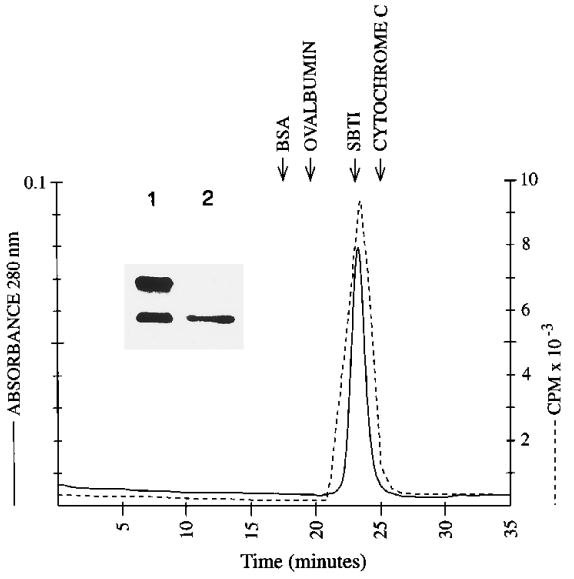

FIG. 7. **Molecular weight determination of the fatty acid-Ch21 complex on Superdex 75 HPLC column.** After incubation with radioactive linoleic acid the isoform A of the recombinant Ch21 was analyzed as described under "Experimental Procedures." *BSA*, bovine serum albumin; *SBTI*, soybean trypsin inhibitor. *Inset* shows analysis of sample by native gel electrophoresis. *Lane 1*, control Ch21, silver-stained. *Lane 2*, protein in the chromatographic peak, autoradiography.

trophoresis in non-reducing conditions. The finding that the ligand binds to the monomeric form of the protein is in agreement with the idea that the binding occurs at an internal hydrophobic pocket as in the case of other members of the family (23).

Ch21 Endogenous Ligand—To gain conclusive evidence to the hypothesis that the Ch21 protein is indeed a specific fatty acid-binding protein, we carried out a series of experiments aimed at identifying the endogenous ligand(s) for this protein. The recombinant protein was purified on a Superdex 75 column and then extracted with ethyl acetate as described above. The dried organic phase was dissolved in methanol and submitted to methylation by reaction with CH_2N_2 for 15 min at room temperature. The methylated extract was then analyzed by gas chromatography or by electron impact mass spectrometry. The gas chromatogram revealed the presence of four major fatty acid methyl esters: palmitoyl- (C16:0, 41.7%), palmitoleoyl- (C16:1, 8.9%), linoleoyl- (C18:2, 13.3%), and stearoyl- (C18:0, 36.1%) methyl esters. The electron impact mass spectrometry spectrum of the methylated extract confirmed this identification by displaying the molecular ion peaks and fragmentation patterns typical of the two most abundant fatty acid methyl esters, *i.e.*, palmitoyl-methyl ester (molecular ion at $m/z = 270$, loss of acetyl or methoxy groups at $m/z = 211$ and 239, aliphatic fragmentation peaks at $m/z = 227$, 199, and 157) and stearoyl-methyl ester (molecular ion at $m/z = 298$, loss of acetyl- or methoxy-groups at $m/z = 239$ and 267, aliphatic fragmentation peaks at $m/z = 255$, 227, and 185). Small peaks at $m/z = 296$ and 294, corresponding to oleoyl- and linoleoyl-methyl esters, respectively, were also observed. The composition of the fatty acids bound to the recombinant Ch21 protein might have reflected the fatty acid composition of baculovirus culture medium. Fatty acids are present in the commercial medium, but their nature and relative concentrations are not available due to patent protection.

To provide data on the endogenous ligand of the protein under more physiologically relevant conditions, *i.e.* in cultures of hypertrophic chondrocytes, we performed some labeling studies by incubating these cells with [U-^{14}C]linoleic acid in the absence of fetal calf serum in linoleic acid-free culture medium. We used this tracer bearing in mind that linoleic acid, among the fatty acids bound to the recombinant Ch21 protein (see above), is the one with the highest affinity for this protein. 18 h incubation of chondrocytes with 0.5 μCi/ml [^{14}C]linoleic acid (2 μM) led to the finding of 27.5% and 64.9% of total incorporated radioactivity in the phospholipid-bound and tri-/diglyceride-bound fatty acid pools, respectively, while only 7.5% of the total radioactivity was due to free linoleic acid. Incorporation of radioactivity (0.1% of total incorporated radioactivity) was also observed in the Ch21 protein purified by Superdex 75 chromatography. The protein was extracted with ethyl acetate, and the extract co-injected with non-radioactive linoleic acid standard onto a reverse-phase HPLC column eluted under conditions ensuring a separation of at least 2 min between even long chain fatty acids or their derivatives (46). A single peak of radioactivity with retention time identical to that of linoleic acid was found, thus showing that the radioactivity bound to the Ch21 protein was due to the binding of linoleic acid.

Detection of Ch21 Protein in Chicken Serum

We have shown previously that the Ch21 is expressed also by cell types other than cartilage and bone cells, including granulocytes (22). We therefore investigated by Western blot for the presence of the protein in the chicken serum (Fig. 8). The specific antibodies recognized in the serum a protein with the same electrophoretic mobility of the purified recombinant Ch21 and of the Ch21 present in the conditioned medium of hypertrophic chondrocytes. No cross-reacting protein was observed in control bovine serum.

DISCUSSION

In this study we have demonstrated that Ch21, a low molecular weight protein of the lipocalin family expressed by hypertrophic chondrocytes *in vitro* and *in vivo* (19–22), binds fatty acids, that the binding is saturable, and that the protein binds ligands as a monomer with a K_d of the order of 10^{-7} M. Long-chain unsaturated fatty acids are preferential ligands. Short-chain fatty acids and long-chain aldehyde and alcohol do not displace the bound long-chain fatty acids. These findings raise the question of the importance of fatty acids in cartilage development and bone formation.

The cascade of events leading to endochondral bone formation from an entirely cartilaginous tissue is not completely

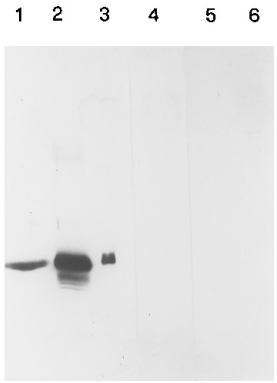

FIG. 8. **Western blot analysis of proteins in chicken serum.** *Lane 1*, control medium of cultured chicken hypertrophic chondrocytes. *Lane 2*, purified recombinant Ch21. *Lanes 3* and *5*, chicken serum. *Lanes 4* and *6*, bovine serum. *Lanes 1–4* were detected with purified antiserum against Ch21. *Lanes 5* and *6* were detected with preimmune serum.

known, although some information is available. Hypertrophic chondrocytes play an active role in bone formation both for the deposition of the first bone (10–14) and because they synthesize and secrete diffusible factors promoting angiogenesis, and possibly stimulating growth and differentiation of surrounding osteoblasts and preosteoblasts (16–18).

The presence of lipids has been shown in cartilage, in particular in cytoplasmic dense bodies of epiphyseal chondrocytes, in the pericellular space, and in calcifying matrix (47), but the role of these lipids and their significance remain unclear. The presence of free fatty acids released by the action of phospholipase A_2 from plasmamembrane phospholipids of growth plate chondrocytes has also been described (48). Vitamin D derivatives modulate phospholipase A_2 activity in cartilage. Phospholipase A_2 activity is required for fatty acid turnover and is the rate-limiting step in production of prostaglandins and other arachidonic acid derived metabolites. A molecule derived from arachidonic acid following the action of lipoxygenase, the 5*S*,12*S*-diHETE, has been identified in osteoblasts and epiphyseal chondrocytes (49). Although in articular chondrocytes synthesis and response to prostaglandins, molecules derived from arachidonic acid following the action of cyclooxygenase, have been reported by several authors, little information is available on prostaglandins in growth plate chondrocytes. Indirect evidence for the presence of prostaglandins in calcified cartilage has been obtained by Okiji *et al.* (50), who have demonstrated by immunohistochemistry expression of prostaglandin-1,2-synthase by chondrocytes in demineralized sections of rat tissues.

Prostaglandin E_1 induces angiogenesis in an *in vivo* assay (51). It is interesting to note that Harada *et al.* (52) have reported potent stimulation of bone formation in cell cultures following increased expression of the angiogenic factor VEGF induced by prostaglandin E_1 and E_2.

It is tempting to speculate that Ch21 has a function in the transport of free fatty acids released from membrane phospholipids or storage lipids. Due to their hydrophobicity, fatty acids cannot diffuse freely in the hydrophilic extracellular microenvironment. Therefore, the requirement for a carrier protein capable of forming a soluble complex with these molecules and of presenting them to cells may be important to the synthesis of active fatty acid metabolites by the cells. Intracellular fatty acid-binding proteins have been described (32–37), but to our knowledge the Ch21 is the first extracellular protein selectively binding fatty acids described in peripheric tissues. The enrichment of Ch21 in tissues differentiating and/or undergoing morphogenesis could be explained by the increased local requirement of fatty acid metabolites acting as local hormones. Additionally, the production of relatively large amount of Ch21 by peripheric granulocytes could be explained by the requirement of the fatty acid substrates for the synthesis of prostanoids and other active metabolites by inflammatory cells.

It is widely accepted that free fatty acids in blood are transported by albumin (38). Ch21 could represent in blood the high affinity, low capacity, specific binding protein that transports fatty acids to target organs, while albumin could represent a low affinity, high capacity storage protein for fatty acids.

Based on our findings, we propose to rename the Ch21 protein as extracellular fatty acid-binding protein (Ex-FABP).

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The Developmentally Regulated Avian Ch21 Lipocalin Is an Extracellular Fatty Acid-binding Protein

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