Developmentally Regulated Synthesis of a Low Molecular Weight Protein (Ch 21) by Differentiating Chondrocytes

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Abstract. When transferred to suspension culture on agarose-coated dishes, dedifferentiated chick embryo chondrocytes resume the chondrocyte phenotype and continue their maturation to hypertrophic chondrocytes (Castagnola, P., G. Moro, F. Descalzi Cancedda, and R. Cancedda. 1986. *J. Cell Biol.* 102:2310–2317). In this paper we report the identification, purification, and characterization of a low molecular weight protein, named Ch 21, expressed and secreted by in vitro differentiating chondrocytes at a late stage of development. This protein is detectable in the cells after a short pulse labeling and is directly secreted in the culture medium. The Ch 21 protein has a peculiar resistance to limited pepsin digestion; nevertheless it is not collagenous in nature as revealed by its unaltered mobility when isolated from cells grown in the presence of α-α' dipyridyl, its resistance to bacterial collagenase, and its amino acid composition. By metabolic labeling of tissue slices and by immunohistochemistry, we show that in the chick embryo tibia the Ch 21 protein first appears at the boundary of the cone of hypertrophic cartilage and in the newly formed bone between the 6 and 10 d of embryo development and localizes in calcifying hypertrophic cartilage thereafter. The Ch 21 protein synthesized by the cultured chondrocytes is closely related and possibly identical to a 21K transformation-sensitive protein associated to the cell substratum of chick embryo fibroblasts.

IN the developmental pathway leading to the organogenesis of long bones, undifferentiated mesenchymal cells pass sequentially through at least three differentiation stages: (a) committed mesenchymal cells, producing type I collagen and possibly basal level of type II collagen (19); (b) stage I (proliferating) chondrocytes, characterized by the synthesis of a large amount of type II collagen (12, 19, 27); and (c) stage II (hypertrophic) chondrocytes, characterized by the synthesis of type X collagen (6, 17, 23).

To investigate endochondral bone formation at a cellular and molecular level we have developed in the last few years a culture system starting from tibial chondrocytes of early stage chick embryos. Freshly dissociated chondrocytes from tibiae of stage 28–30 (11) embryos assume a fibroblast-like morphology and switch from the synthesis of type II to the synthesis of type I collagen when they are cultured as adherent cells on plastic dishes. When these dedifferentiated cells are transferred to agarose-coated dishes (suspension culture) they resume the chondrocyte phenotype and continue their maturation to single, isolated hypertrophic chondrocytes producing type X collagen (8). This process results in a large increase of the duration of all cell cycle phases and of the number of quiescent and degenerating cells (10). When the dedifferentiated cells are cultured in suspension in the presence of ascorbic acid, a cofactor of collagen hydroxylases, they develop into a tissue resembling hypertrophic cartilage (26).

The in vitro transition from dedifferentiated chondrocytes to hypertrophic chondrocytes is characterized by changes in the expression of collagen genes. In particular during the first week of culture type I collagen mRNA, present in high concentration in dedifferentiated cells, rapidly decreases while type II and type IX collagen mRNAs increase (7); the level of type X collagen mRNA increases more progressively and reaches its maximal value after 3–4 wk. These variations in collagen mRNA levels are due to the enhanced transcriptional activity of the corresponding genes (7). The continuous presence of dimethylsulfoxide in the culture medium specifically inhibits accumulation of type X collagen mRNA (21).

By using this culture system we attempted to detect new markers expressed during chondrocyte development. In this paper we report the identification, purification, and characterization of a small, noncollagenous protein expressed and secreted by in vitro differentiating chondrocytes. By metabolic labeling of tissue slices we show a developmentally regulated appearance of this protein in embryonic tibia in vivo. By making use of polyclonal specific antibodies against the Ch 21 protein, using immunohistochemistry, we confirm the biochemical analyses and we indicate the precise location of Ch 21 protein.

A search of the literature for proteins with similar properties prompted us to suggest a possible identity between the Ch 21 protein and a 21K transformation-sensitive protein associated to the cell substratum of chick embryo fibroblasts (3, 4).

Materials and Methods

Cell Culture

Culture medium was Coon's modified F12 medium (1) lacking ascorbic acid and supplemented with 10% FCS. When indicated ascorbic acid was added at a concentration of 50 µg/ml. Adherent dedifferentiated and differentiating chondrocytes were obtained as in Castagnola et al. (8). Dedifferentiated chondrocytes were obtained by plating freshly dissociated chondrocytes from tibiae of 28–30-stage chick embryos (11) in tissue culture dishes. To induce differentiation, fully dedifferentiated chondrocytes, passaged for 2–4 wk as adherent cells, were transferred in suspension culture on agarose-coated (1% in distilled water) dishes.

When indicated 180 mM DMSO was added to the culture medium at the time dedifferentiated chondrocytes were transferred to suspension culture (21).

In some experiments PMA was added at a concentration of 0.1 µg/ml 24 h before labeling.

Chick embryo fibroblasts were prepared according to reference 22.

Cell Labeling and Gel Electrophoresis

Cell labeling was done after 2-h methionine starvation with 100 µCi/ml [^{35}S]methionine for 2 h in the presence of 50 µg/ml ascorbic acid. When indicated tunicamycin (2 µg/ml) and α-α' dipyridyl (100 µg/ml) were added to culture medium during methionine starvation and the 2-h labeling period.

The pulse-chase labeling experiment was performed essentially as described in reference 5. Chondrocytes were grown for 24 h in the presence of ascorbic acid before labeling; after the 2-h starvation in methionine-free medium, [^{35}S]methionine was added for 30 min at a concentration of 100 µCi/ml, cells were washed and fresh medium containing cold methionine was added for the different chase times.

SDS-PAGE was performed under reducing and nonreducing conditions as described by Laemmli (20) and modified by Bonatti and Descalzi Cancedda (2). The concentration of the polyacrylamide was 12.5%. Densitometric scannings of the autoradiographies were performed by a spectrophotometer (model DU8; Beckman Instruments, Inc., Palo Alto, CA).

Limited Proteolysis of Proteins

Limited proteolysis of proteins was performed at 0°C with 100 µg/ml pepsin (Worthington Biomedical Corp., Freehold, NJ) in 0.5 N acetic acid for 16 h.

Purification of the Ch 21 Protein

Hypertrophic chondrocytes were digested with 1 mg/ml hyaluronidase for 15 min at 37°C, plated, and maintained in culture for 4–5 wk as adherent cells. Culture medium was collected daily from confluent 10-cm dishes and replaced by fresh medium (5 ml per dish) according to reference 22. On odd numbered days of harvest, medium was supplemented by 2% FCS, 100 µg/ml β-aminopropionitrile (BAPN), and 100 µg/ml ascorbic acid. On even numbered days, medium was supplemented by 10% FCS and did not contain BAPN and ascorbic acid. Spent media containing low and high serum concentration were made 5 mM EDTA, 0.3 mM phenylmethanesulfonyl fluoride, and pooled separately. Only the pooled medium with low serum was used for the purification of Ch 21 protein. Ammonium sulfate was added to the spent medium to 30% of saturation; the precipitate formed overnight at 4°C was centrifuged at 10,000 g for 30 min and kept as a source of type X collagen. The supernatant was supplemented with ammonium sulfate 90% of saturation; the precipitate containing the Ch 21 protein was separated by centrifugation and redissolved in 1:10 initial volume of 0.15 M phosphate buffer, pH 7.6, containing protease inhibitors. The solution was extensively dialyzed against 0.5 N acetic acid at 4°C (three changes of dialysis) and the precipitate formed was collected by centrifugation and discarded. The supernatant, containing the Ch 21 protein, was stored at 4°C.

Purification of Ch 21 protein from this supernatant was achieved by two different procedures. Procedure a: The protein solution in 0.5 N acetic acid was supplemented with trace amount of culture medium containing [^{35}S]-methionine-labeled Ch 21 protein, dialyzed against 20 mM Tris HCl pH 8.6 containing 2 M urea, and loaded on to a DE 52 column equilibrated with the same buffer. The elution was performed by a gradient of 0–0.4 M NaCl. Peaks from the column were pooled and aliquots were analyzed by 12.5% PAGE. The pool containing the Ch 21 protein was dialyzed against 20 mM sodium acetate pH 3.6 containing 6 M urea and loaded onto a CM 52 column equilibrated by the same buffer. Elution was performed by a gradient of 0–0.3 M NaCl. A single peak of radioactivity was detected; proteins were

pooled, dialyzed against 0.5 N acetic acid, and lyophilized. The lyophilized material was dissolved in PAGE sample buffer by heating at 60°C for 30 min and loaded onto a preparative 12.5% SDS–polyacrylamide gel. After electrophoretic separation the gel was fixed, dried, autoradiographed, and the band corresponding to the Ch 21 protein was cut and electroeluted according to reference 14.

Procedure b: The Ch 21 protein was purified taking advantage of its resistance to limited pepsin digestion (see Results); the protein solution in 0.5 N acetic acid was supplemented by trace amount of culture medium containing [^{35}S]methionine-labeled Ch 21 protein and digested with 100 µg/ml pepsin at 4°C for 16 h. Undigested proteins were precipitated with 10% TCA at room temperature and collected by centrifugation. Precipitated proteins were suspended in PAGE sample buffer, dissolved by heating at 70°C for 30 min, alchylated with iodacetamide in reducing conditions (2), separated by a preparative 12.5% SDS-PAGE, and electroeluted.

Amino Acid Composition of Ch 21 Protein

Amino acid composition was determined on pepsinized and electroeluted Ch 21 protein after removal of residual SDS and contaminating amino acids according to Konisberg and Henderson (18). Hydrolysis was performed in 6 N HCl, 0.05% mercaptoethanol at 110°C for 24 h in vacuum sealed tubes. Amino acid analysis was performed on a single column Carlo Erba amino acid analyzer.

Labeling of Cartilage Slices

After mechanical removal of the perichondrium, cartilage slices obtained from different regions of tibiae and sterna of chick embryos were incubated at 37°C in methionine-free culture medium supplemented with 0.1% FCS, 50 µg/ml ascorbic acid, and 100 µg/ml BAPN. After 2 h [^{35}S]methionine was added at a concentration of 300 µCi/ml and the incubation was continued for an additional 2 h. The culture medium was then collected and clarified by low speed centrifugation; the cartilage slices were washed with PBS, homogenized, and extracted with 4 M guanidine HCl, 0.05 M EDTA, 0.15 M NaCl at 4°C for 16 h. The extract was dialyzed against three changes of 20–30 vol of 0.1 N acetic acid supplemented with 0.5 µg/ml pepstatine, and clarified by low speed centrifugation.

Preparation of Anti–Ch 21 Antiserum

Rabbits were immunized subcutaneously with the Ch 21 protein electroeluted from preparative SDS-PAGE (14). The protein was mixed in a 1:1 ratio with complete Freund's adjuvant and emulsified in two connecting syringes. Rabbits were boosted five times at 2-wk intervals. Serum was taken 1 wk after the last injection and adsorbed on BSA and on FCS proteins coupled to Sepharose 4B. Specificity of anti–Ch 21 antiserum was verified by immunoprecipitation and immunoblotting experiments. The antiserum selectively precipitated the Ch 21 protein from ^{35}S-labeled spent culture medium from hypertrophic chondrocytes and cartilage slices; no other bands were evident. When tested on spent culture medium from hypertrophic chondrocytes, subjected to electrophoresis on SDS-PAGE, and electroblotted on nitrocellulose filter, the antiserum slightly reacted with a protein of 68K (presumably serum albumin). The adsorption of the antiserum on BSA-Sepharose and on FCS-Sepharose eliminated any residual cross-reaction.

Immunoprecipitation

To specifically immunoprecipitate radioactive proteins, labeled cellular extracts and spent culture media were incubated with normal rabbit serum for 1 h at 4°C; 100 µl of Pansorbin (Calbiochem-Behring Corp., La Jolla, CA; 15) were added to each sample and the incubation continued for a further 30 min. After removing the Pansorbin by centrifugation proteins were incubated with the specific antiserum for 16 h. 100 µl of Pansorbin was added and the incubation continued for 30 min at 4°C. Immunocomplexes were washed three times in PBS containing 1% Triton X-100, 0.1% SDS, and 5 mM EDTA, dissolved in the electrophoresis sample buffer, and analyzed by SDS-PAGE.

Immunofluorescence

6- and 10-d-old chick embryo tibiae and the proximal third of 17-d-old chick embryo tibiae were frozen in liquid nitrogen after embedding in OCT (Miles Laboratories, Inc., Naperville, IL). 5-µm frozen serial sections were cut in a cryostat and placed on polylysine-coated microscope slides. After fixa-

tion for 5 min in chilled methanol (−20°C) and washing in PBS, sections were treated with 1 mg/ml of sheep testis hyaluronidase (type III; Sigma Chemical Co., St. Louis, MO) in PBS for 30 min at 37°C, washed, and challenged for 1 h at room temperature with rabbit antisera against chick type I (Centre de Radioanalyse, Institut Pasteur de Lyon, France), II, and X collagens, chick Ch 21, or preimmune rabbit serum previously absorbed on BSA-Sepharose and FCS-Sepharose columns, all diluted 1:20 in PBS containing 4 mg/ml of goat gammaglobulines (Jackson Immuno Research Laboratories Inc., West Grove, PA). After washing with PBS the sections were challenged for 1 h at room temperature with rhodamine goat anti-rabbit IgG (H and L) (Jackson Immuno Research Laboratories Inc.). The slides were then mounted in 90% glycerol in PBS and observed in a Zeiss Axiophot photomicroscope equipped with epifluorescence illumination. Pictures were taken with a 800/1600 Kodak ektachrome film rated as 800 ASA and developed as 1600. Exposure times were identical for pictures taken from different areas of the same sections and the relative controls.

We have performed experiments by treating the sections with other enzymes including trypsin and protease VII alone or in combination with hyaluronidase in order to evidence possible masked Ch 21 sites. Sections treated with hyaluronidase showed the highest fluorescence intensity in the positive areas of the stained tissue.

Antisera to type II and type X collagens were raised in rabbits using electrophoretically pure proteins and tested for specificity by radioimmunoassay, immunoprecipitation, and immunofluorescence, as will be described in future publications.

Results

We identified a low molecular weight protein (Ch 21) synthesized and secreted by in vitro differentiating chondrocytes.

Dedifferentiated chick embryo chondrocytes were labeled with [³⁵S]methionine after 3-wk culture as adherent cells and at different times after their being transferred to suspension culture on agarose-coated dishes. Cell extracts and proteins released by the cells in the culture medium were analyzed on SDS-PAGE (Fig. 1). The pattern of secreted proteins changed during the process of in vitro differentiation; in particular, as expected, during the culture type X collagen appeared and progressively increased. At about the same time the appearance and the progressive increase of a secreted protein with an apparent molecular weight of 21,000 thereafter named Ch 21 protein, was observed. Antibodies directed against this protein failed to immunoprecipitate it from the cell extract and the culture medium of dedifferentiated chondrocytes.

When the cells were labeled in the presence of tunicamycin or α-α′ dipyridyl the electrophoretic mobility of the Ch 21 protein was not affected, suggesting a lack of glycosylation (Fig. 2); in the same figure a decreased mobility of type X collagen and of an unidentified glycoprotein of a lower molecular weight was observed when the cells were labeled in the presence of respectively α-α′ dipyridyl and tunicamycin.

The Ch 21 protein was resistant to digestion by bacterial collagenase but was completely digested by trypsin and pronase at 37°C; treatment with pepsin did not affect the electrophoretic mobility of Ch 21 protein when performed at
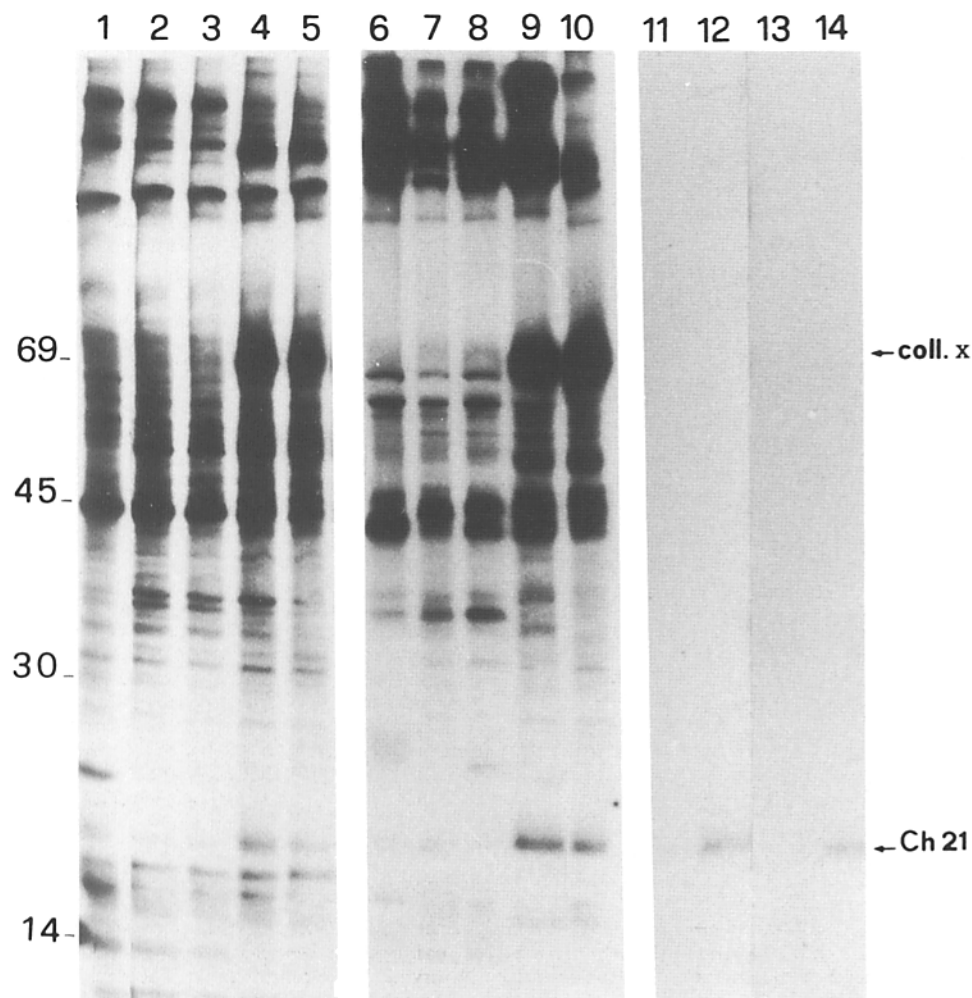
Figure 1. Proteins synthesized and secreted by in vitro differentiating chondrocytes. Intracellular proteins and proteins released by the cells in the culture medium at different times after being transferred to suspension culture were analyzed on 12.5% SDS-PAGE. Lanes *1–5*, intracellular proteins. Lanes *6–10*, secreted proteins. Lanes *1* and *6*, dedifferentiated chondrocytes passaged for 3 wk as adherent cells. Lanes *2–5* and *7–10*, dedifferentiated chondrocytes transferred on agarose-coated dishes and cultured in suspension for 2, 4, 8, and 16 d. Lanes *11–14*, the same samples of lanes *1, 5, 6* and *10* were immunoprecipitated with antibodies against the Ch 21 protein before the electrophoresis. Numbers on the left refer to migrations of molecular weight markers. Equivalent amounts of cell extracts and culture media were loaded on each lane. The immunoprecipitation was performed on half of those amounts.
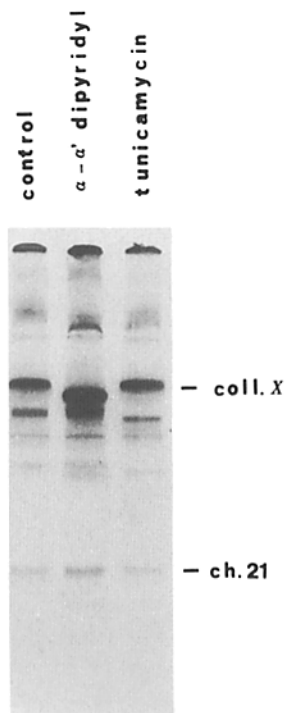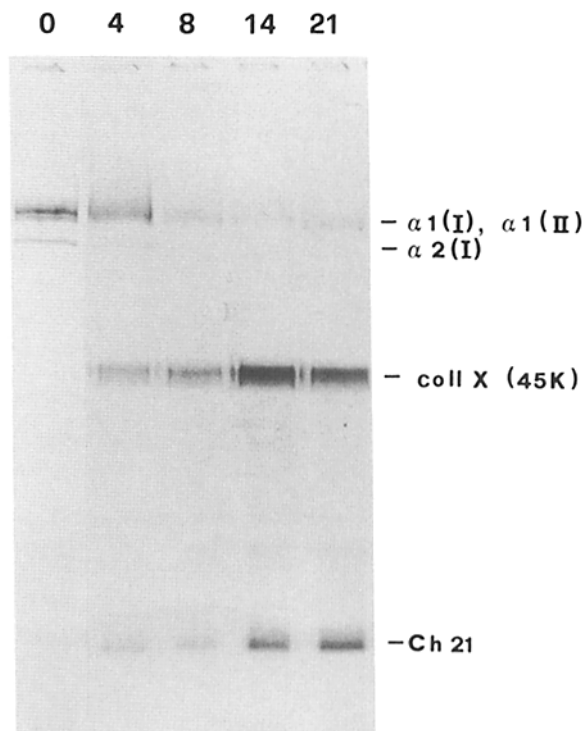
Figure 2. Electrophoretic mobilities of proteins synthesized and secreted by hypertrophic chondrocytes in the presence of α-α' dipyridyl or tunicamycin. Chondrocytes were labeled 11 d after their transferring in suspension culture.

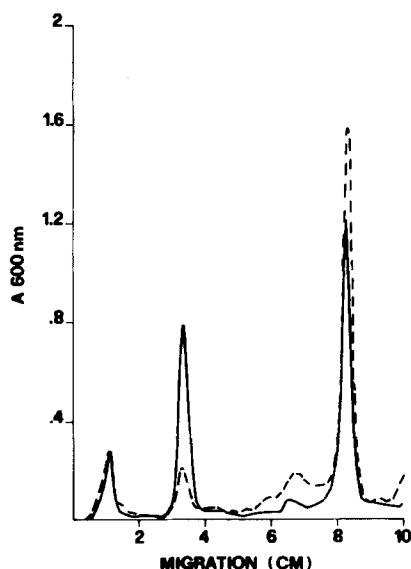Figure 3. Pepsin-resistant proteins secreted by in vitro differentiating chondrocytes. Radioactive proteins released in the culture medium by dedifferentiated chondrocytes (lane 0) and by the same cells transferred in suspension culture for 4, 8, 14, and 21 d were analyzed on SDS-PAGE after pepsin digestion.

37° or 0°C (not shown). The Ch 21 protein presents a lower electrophoretic mobility under reducing conditions; this suggests the existence of intrachain disulfide bonds in the native molecule (not shown). Taking advantage of the resistance of Ch 21 protein to limited pepsin digestion the appearance of this protein during the in vitro differentiation of chondrocytes was further investigated. Pepsin-resistant proteins secreted by the cells at different times after transfer into suspension culture are shown in Fig. 3; only helical portions of collagens and Ch 21 protein are detectable. Once again a switch from the synthesis of type I to the synthesis of type II collagen is observed in the 1-wk culture; the switch is followed by a progressive decrease of type II collagen and by a progressive increase of type X collagen. The increase of the Ch 21 protein is almost concomitant to the increase of type X collagen. Nevertheless certain data suggest that the synthesis of the type X collagen and the Ch 21 protein is under the control of different regulatory mechanisms. At variance with the increase of type X collagen, the increase of Ch 21 protein was not affected by the presence of 180 mM DMSO during the culture (Fig. 4); immunofluorescence staining with specific antibodies show a different location of the two proteins in embryonic cartilage slices (see below).
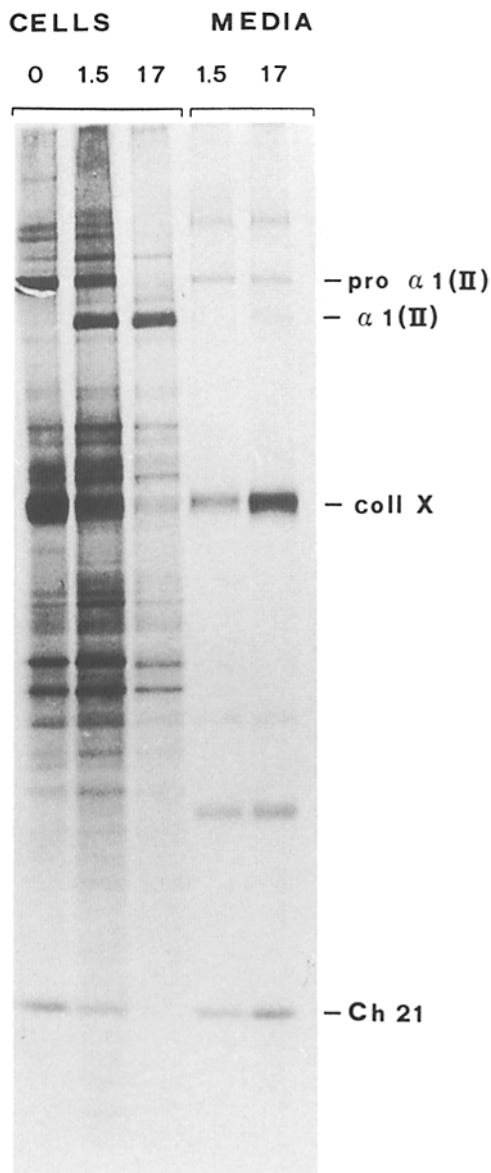
Pulse-Chase Labeling of Chondrocytes

The biosynthesis of Ch 21 protein and its secretion were investigated by pulse labeling of hypertrophic chondrocytes (Fig. 5). The Ch 21 protein was clearly detectable in the cell lysates after 30-min pulse, was still present after 90-min chase, and disappeared after 17-h chase. The disappearance of the Ch 21 protein from the cells was paralleled by its contemporary progressive appearance in the medium. These results strongly argue against the presence of a larger molecular weight precursor for this protein.

Figure 4. Densitometric scanning of pepsin resistant protein secreted by chondrocytes grown in the presence and in the absence of 180 mM DMSO. Cells were labeled 17 d after transferring in suspension culture and secreted proteins were pepsin digested and analyzed on SDS-PAGE. (———) Proteins from chondrocytes grown in the absence of DMSO. (- - - -) Proteins from chondrocytes grown in the presence of DMSO. The same amount of counts was applied to each lane in the polyacrylamide gel.

CELLS   MEDIA

0   1.5   17   1.5   17

—pro α 1(Ⅱ)

— α 1(Ⅱ)

— coll X

—Ch 21

Figure 5. Pulse-chase labeling of chondrocytes. Hypertrophic chondrocytes growing as adherent polygonal cells were pulse labeled with [³⁵S]methionine for 30 min. At the end of the pulse period (*0*) and after 1.5- and 17-h chase the medium was collected and the cell layer solubilized. Equivalent volumes of cell extracts and culture media were analyzed on SDS-PAGE. Further details are given in the text.

Purification of the Ch 21 Protein from Culture Medium

The purification of the Ch 21 protein from spent culture medium was achieved by the procedures described in Materials and Methods. In Fig. 6 the chromatographic separation of proteins on the DE52 column (procedure a) is shown. When analyzed on polyacrylamide gel, after silver stain both the native Ch 21 protein purified according to procedure a and the Ch 21 protein purified after limited pepsin digestion according to procedure b were electrophoretically homogeneous (not shown).

The amino acid composition of the Ch 21 protein purified according to procedure b is reported in Table I.

Developmental Appearance of the Ch 21 Protein in the Chick Embryo Tibia

Cartilage slices were obtained from 6-d-old embryo whole tibiae and from growth plate of 17-d-old embryo tibiae and incubated with [³⁵S]methionine. The presence of Ch 21 protein in the radioactive proteins released in the incubation medium was investigated by immunoprecipitation with the specific antibodies (Fig. 7); the Ch 21 protein was immunoprecipitated from medium of slices of 17-d-old tibia growth plate also containing type X collagen (lanes 2 and 4) but not from medium of slices of 6-d-old tibiae that did not contain type X collagen (lanes 1 and 3). Similar results were obtained when the radioactive proteins extracted from the tibia cartilage after the incubation were analyzed (not shown).

To further investigate the developmental appearance of the Ch 21 protein in the chick embryo tibial cartilage, frozen sections of tibiae from 6-, 10-, and 17-d-old embryos were analyzed by immunohistochemistry with specific antibodies.

6-d-old Embryo Tibia. Ch 21 was not present. Fig. 8, *a* and *b* show that type II collagen is clearly present in the cartilage while no Ch 21 is observed. As expected at this stage of development type X collagen was not detected (not shown).

10-d-old Embryo Tibia. Ch 21 was observed in the proximal third of the diaphysis at the border between hypertrophic cartilage cone and the newly formed bone tissue (Fig. 9, *a* and *a'*). The same area stained for type I collagen (Fig. 9, *b* and *b'*).

17-d-old Embryo Tibia. At this stage of bone development Ch 21 was found in the hypertrophic cartilage, positive to type X collagen staining (Fig. 10, *c*) next to the bone tissue, around lacunae of vascular erosion and in the bone itself (Fig. 10 *a*). Type I collagen was present in the same areas although its distribution was more discrete (Fig. 10 *b*).

Possible Identity between the Ch 21 Protein and a 21K Transformation-sensitive Protein Associated with the Extracellular Matrix of Chick Embryo Fibroblasts

A search of literature for a protein with properties similar to the Ch 21 protein brought to our attention a 21-kD transformation-sensitive protein secreted by chick embryo fibroblasts and inducible by treatment with PMA (3, 4). To investigate a possible relationship between Ch 21 and this protein, chick embryo fibroblasts and control dedifferentiated chondrocytes were labeled with [³⁴S]methionine before and after treatment with PMA for 24 h. When the radioactive proteins were analyzed, a protein with the same electrophoretic migration of the Ch 21 protein was present in the culture medium of the fibroblasts; this protein significantly increased in the cells treated with PMA (Fig. 11 *a*). The antibodies raised against the Ch 21 protein also immunoprecipitated the 21K transformation-sensitive protein secreted by the fibroblasts; a low amount of a protein of 21,000 mol wt was detected by the antibodies also present in the culture medium of the dedifferentiated chondrocytes treated with PMA (Fig. 11 *b*).

Discussion

Here we report the synthesis of a low molecular weight protein, named Ch 21, by cultured chick embryo chondrocytes. The expression of this protein, which is not made by
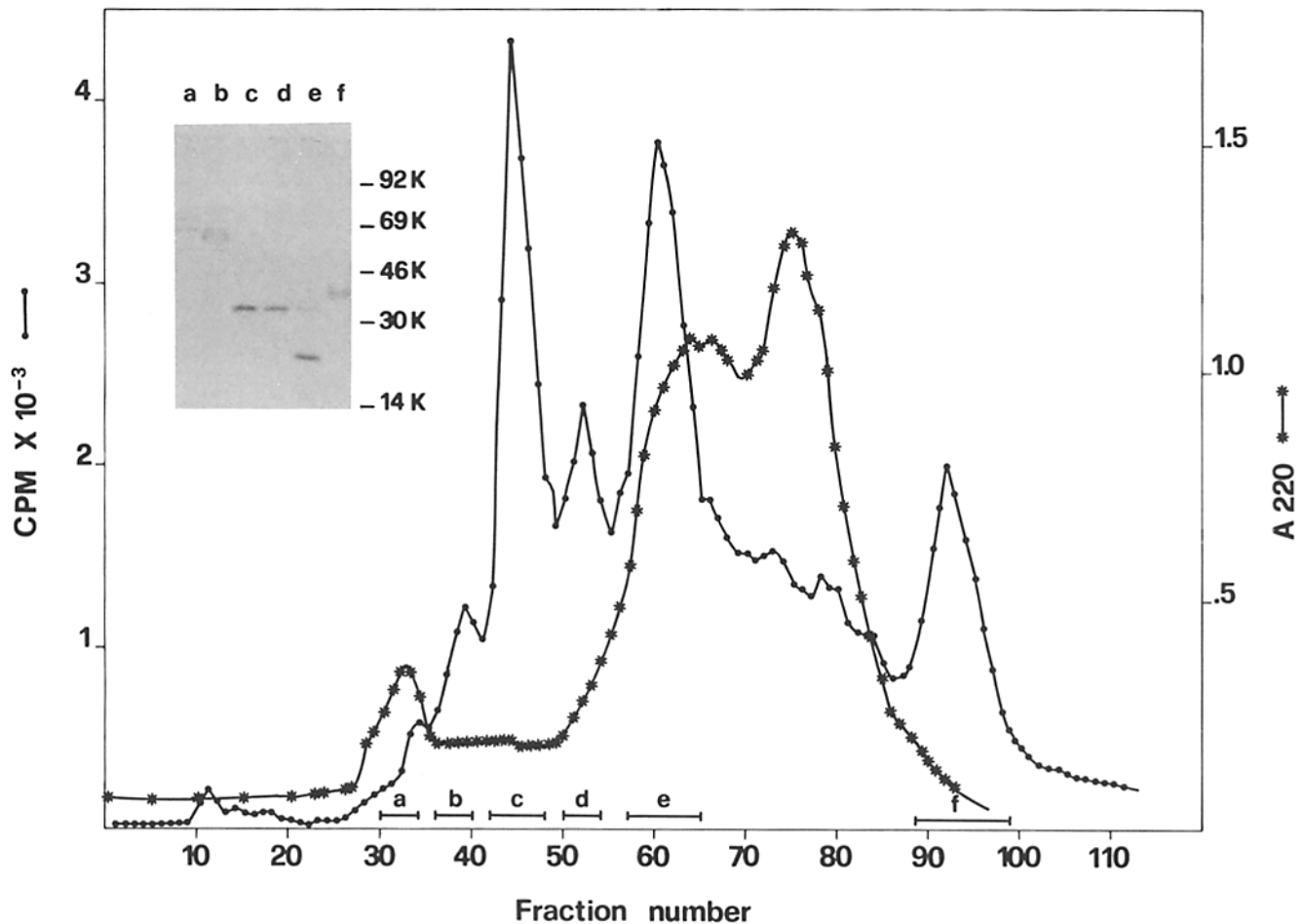
Figure 6. Chromatographic separation of the Ch 21 protein on a DE52 column. An ammonium sulfate precipitate from spent culture medium of hypertrophic chondrocytes was applied to a DE52 column equilibrated with 20 mM Tris HCl pH 8.6 containing 2 M urea. Elution was performed by a 0–0.4 M NaCl gradient. Further details are given in Materials and Methods. (*Inset*) SDS-PAGE of the pooled peaks from the column. Numbers on the right refer to the electrophoretic migration of molecular weight markers.
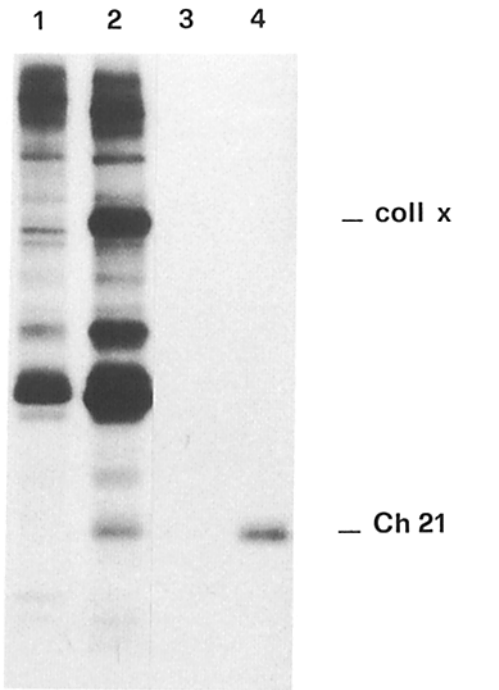
Table I. Amino Acid Composition of the Ch 21 Protein

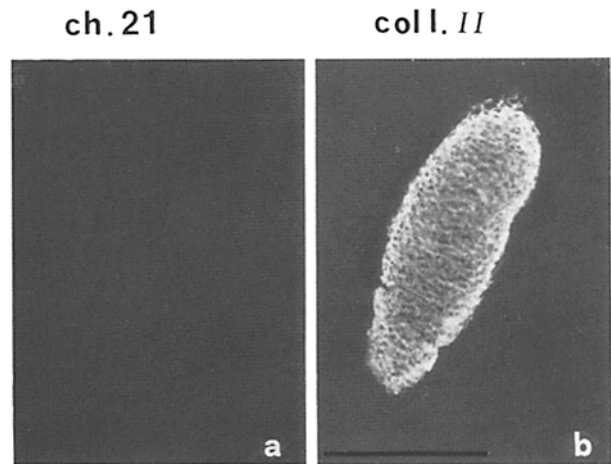| Amino acid	Residues per mole
CM Cys	7
Asp	20
Thr	11
Ser	13
Glu	26
Pro	21
Gly	13
Ala	22
Val	16
Met	2
Ile	6
Leu	20
Tyr	4
Phe	9
His	6
Lys	10
Arg	7

The amino acid composition is the average of two determinations on the same preparation.

dedifferentiated chondrocytes grown as adherent cells, is activated by transferring the cells in suspension culture; its synthesis continuously increases during the in vitro differentiation to hypertrophic chondrocytes and approximately parallels the increase in the synthesis of type X collagen. Nevertheless the expression of the Ch 21 protein and the type X collagen are probably under the control of different regulatory mechanisms.

The Ch 21 protein is not a mannose-containing glycoprotein; its electrophoretic migration does not change when the cells are labeled in the presence of tunicamycin. When we tested the sensitivity of the Ch 21 protein to different proteases, we found a peculiar resistance of the protein to limited pepsin digestion. Nevertheless it was not collagenous in nature as revealed by its unaltered mobility when isolated from cells grown in the presence of α-α' dipyridyl, its resistance to bacterial collagenase and, more important, its amino acid composition determined on the Ch 21 protein purified from culture medium. The amino acid composition of the Ch 21 protein revealed, in accordance with the observed presence of intramolecular disulfide bonds, a comparatively high

Figure 8. Immunofluorescence staining with anti–Ch 21 protein (a) of an oblique section trough the mid-diaphysis of a 6-d-old chick embryo tibia. No presence of this protein is observed. A subsequent serial section stained with anti–type II collagen antibodies (b) shows the presence of cartilagenous tissue in the area. Bar, 800 μm.

Figure 7. Analysis of labeled proteins released in the culture medium by slices from 6-d-old whole tibiae (lane 1) and from growth plate of 17-d-old tibiae (lane 2). In lanes 3 and 4 the same samples of lanes 1 and 2 were immunoprecipitated with antiserum against the Ch 21 protein before electrophoresis.
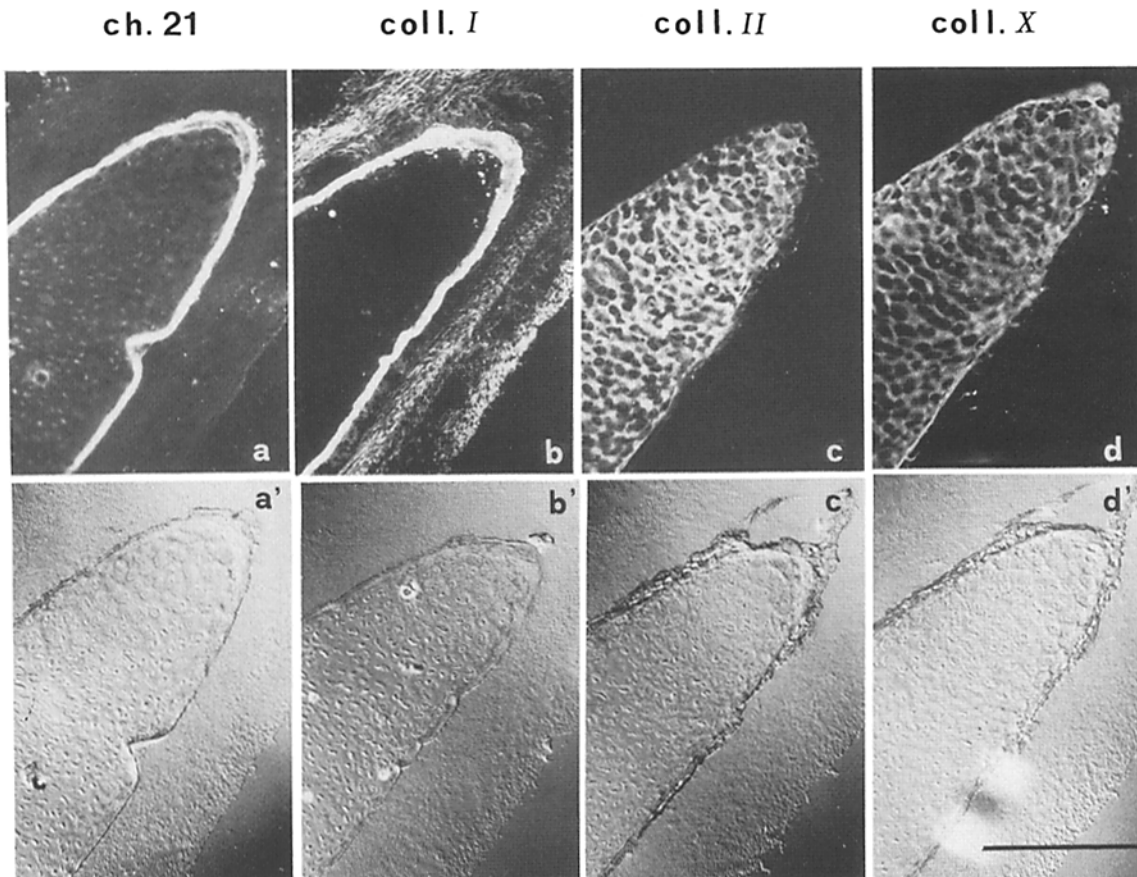
Figure 9. Localization of the Ch 21 protein by immunofluorescence in 10-d-old chick embryo tibiae. In this tissue Ch 21 has been found only at the boundary between hypertrophic cartilage and the newly formed bone spicule and in the bone tissue itself (a). Type I collagen shows a similar distribution in a contiguous serial section and it is, in addition, localized in the muscular and mesenchymal tissue surrounding the cartilagenous cone (b). The cartilage in this area contains both type II (c) and type X (d) collagens. The latter kind of collagen is characteristic of hypertrophic cartilage. a', b', c', and d' show the same fields as in a, b, c, and d but viewed with interferential contrast illumination. Bar, 400 μm.
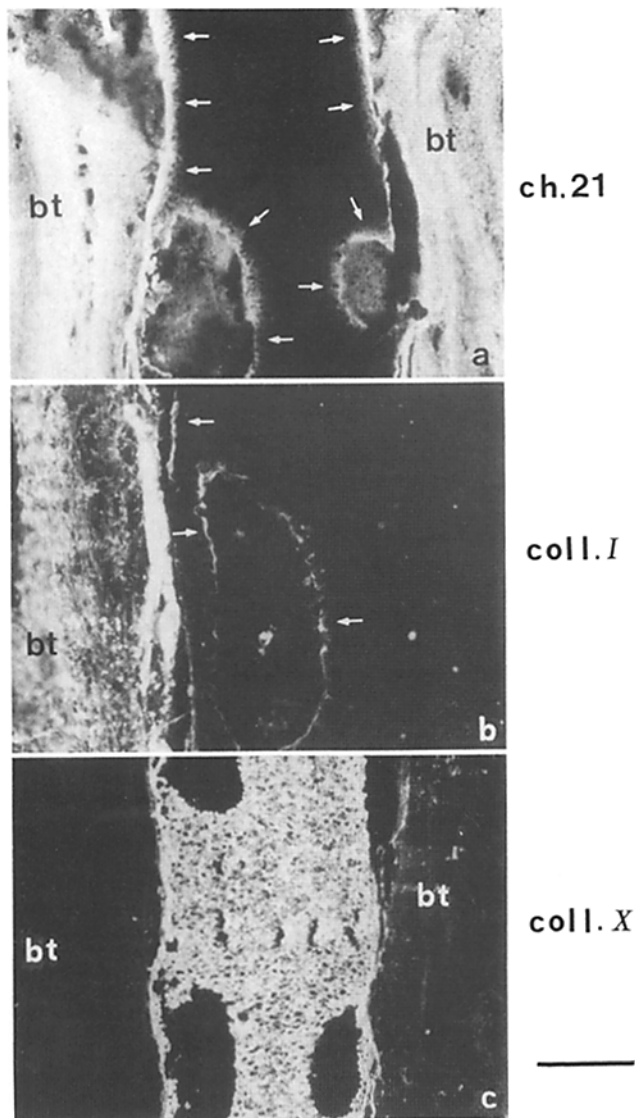
Figure 10. Immunofluorescence localization of Ch 21 protein in the hypertrophic region of 17-d-old chick embryo tibia. (*a*) Ch 21 is present (*arrows*) in the peripheral layer of hypertrophic cartilage at the border of bone tissue and around lacunae of vascular erosion; bone tissue is also positive for Ch 21 (*bt*). (*b*) Type I collagen is localized in the same areas as Ch 21 (*arrows*), although type I collagen has a more discrete distribution pattern than Ch 21 (compare *a* and *b*). (*c*) The presence of type X collagen characterizes this area as hypertrophic. *a*, *b*, and *c* are pictures taken from corresponding areas on serial sections of the same specimen. *b* is shown at a higher enlargement than *a* and *c* to better demonstrate the fine localization of type I collagen. Bars: (*a* and *c*) 200 µm; (*b*) 115 µm.

percentage of cysteines. A computer-assisted fast scan for similarities between known protein sequences and preliminary amino acid sequences derived from the purified Ch 21 protein did not reveal any significant homology.

The Ch 21 protein is already detectable in the cells after a short pulse labeling time and is secreted as such in the culture medium. The observation that specific antibodies raised against the Ch 21 protein failed to recognize any larger molecular weight precursor in the cells is in agreement with this finding.

At a later time of the culture the Ch 21 protein is synthesized in greater quantity and becomes one of the major products secreted by the cells; in this paper we describe its purification in quantity of the order of 100 µg/liter starting from spent culture medium of hypertrophic chondrocytes.

In the chick embryo, by metabolic labeling of tissue slices and by immunohistochemistry, we have shown the presence of the Ch 21 protein in the zone of hypertrophic cartilage of tibia where erosion of cartilage and formation of bone occurs. In particular we have reported that in the tibia the Ch 21 protein first appears between 6 and 10 d of embryo development at the boundary of the cone of hypertrophic cartilage and in the newly formed bone tissue.

Antibodies directed against Ch 21 protein also heavily stained the extracellular matrix of newly formed bone. Synthesis in vitro of radioactive Ch 21 protein by cultured chick embryo bone slices has been observed (Manduca, P., F. Descalzi Cancedda, C. Tacchetti, R. Quarto, P. Fossa, and R. Cancedda, manuscript in preparation). These findings may reflect the common origin of chondrogenic and osteogenic lineages. It must be recalled that, although the hypothesis is very controversial, a conversion of hypertrophic chondrocytes to osteoblasts has been reported (16).

Although much information is available on structure and location of the Ch 21 protein, we do not know anything about its function. Therefore we can only speculate, mainly on the basis of its location in the extracellular matrix, about a possible interaction with other extracellular matrix macromolecules. The possibility of a role of the Ch 21 protein as a growth factor may be considered. It is secreted by the cells and it may be associated with the extracellular matrix, it has a molecular weight in the range of the molecular weight of several known growth factors, it has internal disulfide bonds and, more importantly it appears developmentally regulated (15). Further investigations are necessary to verify this possibility.

Our data indicate a close relationship and a possible identity between the Ch 21 protein and the 21K transformation-sensitive protein described by Blenis and Hawkes (3). The two proteins have the same molecular weight, are recognized by the same antibodies, are secreted and may be associated with the extracellular matrix, are not mannose-containing glycoproteins, have intrachain disulfide bonds, and share a peculiar resistance to limited proteolytic digestion. Blenis and Hawkes have suggested that increased synthesis and deposition in the extracellular matrix of the 21K protein are general characteristics of the early stages of fibroblast transformation (3); we have found that the Ch 21 protein is synthesized in a large amount by normal chondrocytes. Although the real significance of this observation has to be further investigated, it is interesting to note that once more normal chondrocytes seem to share a property believed to be characteristic of transformed cells, other properties being: anchorage independent growth (8, 13, 28), capacity to form colonies in soft agar (13), presence on the cell surface of glycoproteins observed in transformed but not in normal fibroblasts (9).

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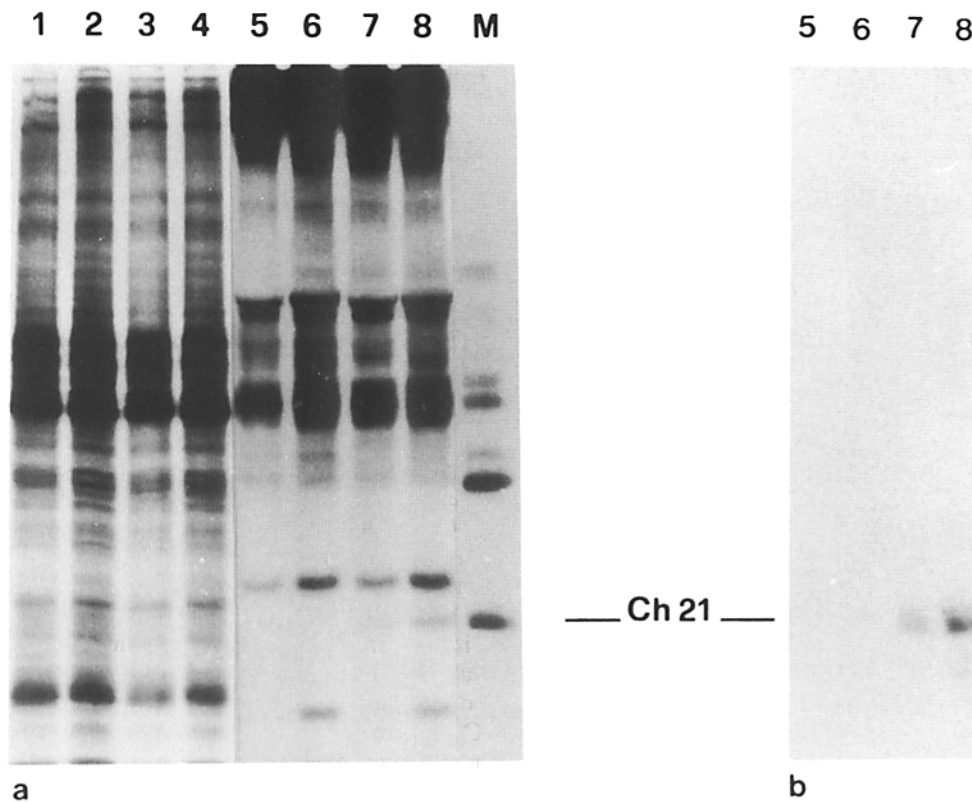
Figure 11. Comparison between the Ch 21 protein and the 21K transformation-sensitive protein of chick embryo fibroblasts. Radioactive proteins from dedifferentiated chondrocytes (lanes *1, 2, 5,* and *6*) and from chick embryo fibroblasts (lanes *3, 4, 7,* and *8*) associated to the cell layer (lanes *1–4*) and released in the culture medium (lanes *5–8*) were analyzed on SDS-PAGE. The cells were cultured under standard culture conditions (lanes *1, 3, 5, 7*) or treated with PMA for 24 h before labeling (lanes *2, 4, 6,* and *8*). In lane *M* a partially purified culture medium from hypertrophic chondrocytes containing radioactive Ch 21 protein was applied. In *b* a double amount of samples applied in lanes *5–8* of *a* were digested with pepsin and immunoprecipitated with anti–Ch 21 specific-antiserum before electrophoresis.

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