Purification of a Cartilage-derived Growth Factor*

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A growth factor was isolated from bovine cartilage and purified by a combination of gel filtration chromatography and isoelectric focusing. In the first step of the purification, growth factor activity as measured by stimulation of DNA synthesis in quiescent 3T3 cells or chondrocytes, was extracted by incubation of cartilage with 1 M guanidine hydrochloride, pH 6.0. The crude cartilage extract was analyzed by gel filtration on a column equilibrated with 4 M guanidine hydrochloride and 5 mM dithiothreitol. All of the cartilage-derived growth factor activity migrated in a region corresponding to molecular weights between 12,000 and 20,000. The low molecular weight fraction was analyzed by isoelectric focusing in the presence of 6 M urea. Two major active fractions were found, with isoelectric points of approximately 9 and 10. Purification to homogeneity was accomplished by several cycles of gel filtration of the PI 9 fraction on columns equilibrated with 4 M guanidine hydrochloride and 5 mM dithiothreitol. The purified growth factor migrated as a single polypeptide band in two polyacrylamide gel electrophoresis systems, sodium dodecyl sulfate polyacrylamide gel electrophoresis and acid-urea polyacrylamide gel electrophoresis. The molecular weight of the cartilage-derived growth factor was estimated to be between 16,000 and 18,000 by gel filtration and 16,400 by SDS polyacrylamide gel electrophoresis. Gel filtration chromatography of the PI 10 fraction also yielded an active purified 16,400 molecular weight growth factor. Approximately 1 to 2.5 μg/ml of purified cartilage-derived growth factor was necessary to half-maximally stimulate DNA synthesis in 3T3 cells and chondrocytes.

In a previous report it was demonstrated that a guanidine extract of bovine scapular cartilage contains a growth-promoting factor. The cartilage-derived growth factor stimulated DNA synthesis and cell division in quiescent 3T3 cells and in bovine scapular chondrocytes (1). A preliminary biochemical characterization showed the cartilage-derived growth factor to be a relatively low molecular weight cationic polypeptide. In addition, the growth factor was not inactivated by 2-mercaptoethanol or by dithiothreitol. This feature distinguished the cartilage-derived growth factor from many of the blood-derived growth factors. For example, the platelet-derived growth factor, nonsuppressible insulin-like activity, multiplication stimulation activity, and somatostatin C are irreversibly inactivated by sulfhydryl-reducing agents (2-5).

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The site of synthesis of the cartilage-derived growth factor has not yet been determined. However, we have found that low molecular weight growth factor activity is present in lysates of freshly isolated chondrocytes prepared by collagenase digestion of bovine scapular cartilage (6). Furthermore, if the chondrocytes are subfractionated into subcellular components, all of the cellular growth factor activity is found to be in the nucleus, and all of the nuclear activity is associated with chromatin (6). In this report we describe the purification of a growth factor from bovine scapular cartilage and demonstrate that it is a polypeptide with a molecular weight of about 16,400.

MATERIALS AND METHODS

DNA Synthesis and Cell Division—Growth factor activity was assayed by measuring stimulation of DNA synthesis and cell division in confluent monolayers of quiescent mouse Balb/c 3T3 cells (clone A31) or second passage bovine scapula chondrocytes. The preparation and culture of primary chondrocytes from cartilage has been previously described (7). The medium used for the growth of the cells was Dulbecco’s modified Eagle’s medium, supplemented with 4.5 g/liter of glucose (Grand Island Biological Co.), 10% calf serum (Colorado Serum Co., Denver, CO), 50 units of penicillin/ml, and 50 μg of streptomycin/ml. The preparation of quiescent monolayers of cells and the methods used to measure DNA synthesis and cell division in the cells have been described previously (1, 8). Most of the bioassays described in this report were performed using the established 3T3 cell line which can be passaged routinely. However, the same results were obtained with second passage chondrocytes (see Fig. 6). Protein concentrations were measured both by the Lowry procedure (9) and by a protein assay procedure using a reagent purchased from Bio-Rad Laboratories (Richmond, CA). The Bio-Rad assay was used when the presence of dithiothreitol interfered with the Lowry assay. Otherwise the two assays gave the same protein values.

Extraction of Cartilage—Growth factor was extracted from bovine scapular cartilage as described previously (1) with modifications. The extracts were kindly provided to us by Dr. James Schuck (Monsanto Company, St. Louis, MO) and were prepared as follows. Four hundred grams of finely scraped calf cartilage were extracted with 4 liters of 1.0 M guanidine hydrochloride buffered with sodium maleate, pH 6.0, for 23 h at room temperature. The extract was filtered and then concentrated 20-fold by ultrafiltration in a pellicon cassette system (Millipore) using a PTGC ultrafilter (10,000 M, cutoff). The concentrated sample was dialyzed overnight against distilled H2O and clarified by centrifugation. The supernatant fraction was filtered through a 0.45-μm membrane (Type HA, Millipore) using a PTGC ultrafilter (10,000 M, cutoff) ultrafilter and lyophilized. Four hundred grams of cartilage yielded approximately 2 g of crude extract. The cartilage extract was also used to purity a cartilage-derived trypsin inhibitor (10). Some of the extracts used in the purifications described in this report were pretreated by passage over a Sepharose-trypsin affinity column (10) to remove the trypsin inhibitor. However, the same purification was achieved whether the extracts were exposed to Sepharose-trypsin affinity chromatography or not.

Gel Filtration Chromatography—Two types of columns were used for gel filtration chromatography. 1) A Bio-Gel A-0.5m column (2.0 × 98 cm) was used for large scale purification of crude cartilage extract. The column was equilibrated with 4 M guanidine hydrochloride, 5 mM dithiothreitol, and 0.02 M 2-(N-morpholino)ethanesulfonic acid.
Polyacrylamide gel electrophoresis—Two polyacrylamide gel electrophoresis systems were used for the analysis of the cartilage-derived growth factor. In both cases, a model 220 vertical slab electrophoresis cell with a 1.5-mm thick slab was used. (a) Polypeptides were analyzed using the sodium dodecyl sulfate electrophoresis system described by Laemmli (12) with modifications. The stacking gel contained 5% acrylamide and 0.1% SDS and had a pH of 8.8. The sample buffer contained 2% SDS and 10% 2-mercaptoethanol and had a pH of 6.8. Samples were heated in a boiling water bath for 5 min prior to electrophoresis. Electrophoresis was carried out for about 21 h at room temperature at a constant voltage of 30 V.

For molecular weight determination, 2.5 μg of standards of known molecular weight were used (Bio-Rad). The standards were phospholipase A2 (Mr = 9,400), ovalbumin (Mr = 43,000), carbonic anhydrase (Mr = 30,000), soybean trypsin inhibitor (Mr = 21,000), and lysozyme (Mr = 14,300). (b) Polypeptides were analyzed using the urea-acetic acid system of Panyim and Chalkley (13). The slab gel contained 15% polyacrylamide and 6.25% urea and had a pH of 3.2. After 24 h, the gel was stained with 0.25% Coomassie blue R250 for 20 min with 0.25% Coomassie blue, and destained for 10 min with 10% acetic acid. The stained and unstained gels were lined up with each other, and the region of the unstained gel containing the growth factor was excised with a scalpel. The gel slice was subsequently incubated overnight at room temperature with 25 μl of 1.0% (w/v) SDS made up in H2O. The eluate was dialyzed against distilled H2O to remove as much SDS as possible. The rest of the SDS was removed by subsequent gel filtration on a Sephadex G-75 superfine column (1.5 x 85.5 cm) equilibrated with 4 M guanidine hydrochloride and 5 mM dithiothreitol.

Amino Acid Analysis—The amino acid composition of purified cartilage growth factor was determined by amino acid analysis with and without performic acid oxidation. About 3 to 6 nmol of polypeptide was re suspended in 6 N HCl and phenol (0.1% w/v in 6 N HCl) and hydrolyzed for 24, 48, or 72 h at 110°C under a vacuum of 0.005 mm Hg. Amino acid analysis using ninhydrin was carried out in 0.005 M phosphate buffer, pH 7.5, to a concentration of 0.5 mM/10 μl. To 10 μl of cartilage-derived growth factor (10 μg/10 μl) were added 68,000, ovalbumin (Mr = 43,000), chymotrypsinogen (Mr = 28,000), myoglobin (Mr = 17,800), lysozyme (Mr = 14,300), insulin (Mr = 5,800) for the intact molecule and Mr ~ 2,900 for the denatured form.

RESULTS

Gel Filtration Chromatography—A cartilage extract was prepared by incubation of bovine scapula cartilage with 1 M guanidine hydrochloride at pH 7 (14). After concentration of approximately 220 μg/ml, the crude extract stimulated half-maximal DNA synthesis in quiescent Balb/c 3T3 cells and in bovine scapula chondrocytes. The crude cartilage extract was analyzed on a gel filtration column equilibrated with 4 M guanidine hydrochloride and 5 mM dithiothreitol, reagents whose cumulative effect is to dissociate proteins to their constituent polypeptide chains (16). When analyzed by col-
Isoelectric Focusing—The low molecular weight growth factor activity (a pool of Fractions 30 to 39) obtained by gel filtration as described in Fig. 1 was analyzed by isoelectric focusing using ampholytes in the pH 3.5 to 10 range. The isoelectric focusing of cartilage-derived growth factor activity is cationic and had a PI between 9 and 10. The remaining growth factor activity had a pI of about 4. The pI 4 material was part of a precipitate near the bottom of the column and probably does not represent a true fractionation. Another aliquot from the Fraction 30 to 39 pool of the gel filtration column in Fig. 1 was analyzed by isoelectric focusing using ampholytes in the pH 9 to 11 range in order to increase resolution in the high pH range (Fig. 3). Two peaks of growth factor activity were found, one with a pI of about 9 and another with a pI of about 10. Both peaks were analyzed further.

Gel Filtration Chromatography and Analysis by Polyacrylamide Gel Electrophoresis—The fraction with a pI of 9 shown in Fig. 3 was further purified by chromatography on Sephadex G-75 equilibrated with 4 M guanidine hydrochloride and 5 mM dithiothreitol. The active fractions of the G-75 column were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The cartilage-derived growth factor activity was found mainly in Fraction 17 of the Sephadex G-75 column and to a lesser extent in Fraction 18 (Fig. 4, left). Gel filtration of polypeptides in the presence of guanidine hydrochloride and dithiothreitol is considered to be a sensitive measure of molecular weight (16). The molecular weight of the cartilage-derived growth factor was estimated by this technique to be between 16,000 and 18,000. Analysis of the polypeptides in Fraction 17 by SDS-PAGE revealed that over 95% of the protein was in one polypeptide (Fig. 4, right, slot 3). SDS-PAGE is also a sensitive measure of polypeptide molecular weight. Using proteins of known molecular weight (slot 1), the molecular weight of the major polypeptide band (arrow) was estimated to be about 16,400. Furthermore, the relative distribution of this 16,400 M, polypeptide on the slab gel correlated well with the distribution of growth factor activity on the column. It was concluded that the 16,400 M, polypeptide was most likely to be the cartilage-derived growth factor.

A similar analysis involving Sephadex G-75 chromatography and SDS-PAGE was conducted with the active fraction in Fig. 3 having a pI of 9. The growth factor activity of the pI 10 fraction had a molecular weight between 16,000 and 18,000 on the Sephadex G-75 column (Fig. 5, left). A 16,400 M, polypeptide band (arrow) was found in the corresponding SDS-PAGE analysis of Fractions 19, 20, and 21 (Fig. 5, right), whose relative distribution on the slab gel correlated well with the distribution of growth factor activity on the column.

Purification of the Cartilage-derived Growth Factor to Homogeneity—Purification of the cartilage-derived growth factor was estimated by this technique to be between 16,000 and 18,000. Analysis of the polypeptides in Fraction 17 by SDS-PAGE revealed that over 95% of the protein was in one polypeptide (Fig. 4, right, slot 3). SDS-PAGE is also a sensitive measure of polypeptide molecular weight. Using proteins of known molecular weight (slot 1), the molecular weight of the major polypeptide band (arrow) was estimated to be about 16,400. Furthermore, the relative distribution of this 16,400 M, polypeptide on the slab gel correlated well with the distribution of growth factor activity on the column. It was concluded that the 16,400 M, polypeptide was most likely to be the cartilage-derived growth factor.

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factors to homogeneity could be accomplished by repeated gel filtration of active fractions with either a pI of 9 or a pI of 10. For example, highly purified growth factor with a pI of 9 (Fig. 4, Fraction 17) was applied to a Sephadex G-75 column equilibrated with 4 M guanidine hydrochloride and 5 mM dithiothreitol. The fractions eluting from the column were tested for their ability to stimulate DNA synthesis in both Balb/c 3T3 cells and in bovine scapula chondrocytes (Fig. 6).

As measured in both cell types, all of the growth factor activity was found to migrate in the same fraction corresponding to a molecular weight of about 16,000 to 18,000. Thus, the same cartilage-derived growth factor stimulated DNA synthesis in chondrocytes as well as in 3T3 cells. The homogeneity of this cartilage-derived growth factor preparation was demonstrated by analysis of the banding pattern in two polyacrylamide gel electrophoresis systems, the SDS-PAGE system which separates polypeptides on the basis of molecular weight (12) and the acid-urea gel system (urea-PAGE) described by Panyim and Chalkley (13) which separates polypeptides mainly on the basis of charge and which was designed for the analysis of cationic proteins such as histones. A densitometer trace of purified growth factor analyzed by SDS-PAGE (inset, slot 1) is shown in Fig. 7. Only one polypeptide is present in the preparation. A densitometer trace of the same purified growth factor (inset, slot 2) analyzed by urea-PAGE is shown in Fig. 8. Again, only one polypeptide is present. When compared to histones, it was found that the cartilage-derived growth factor migrated slightly more slowly than did the histone H1. In 15 SDS-PAGE experiments, each representing different preparations of cartilage-derived growth factor, the molecular weight was calculated to be 16,400 with a standard deviation of 356 and a standard error of the mean of 92.

Growth factor activity (Fig. 5, Fraction 20) with a pI of 10 was also purified by repeated gel filtration on Sephadex G-75 in the presence of 4 M guanidine hydrochloride and 5 mM dithiothreitol. An SDS-PAGE analysis of the purified cartilage-derived growth factor (Fig. 9, slot 2) indicates the presence of a single polypeptide band with a molecular weight of 16,400 (arrow). It is apparent that the two active peaks with a pI of 9 and a pI of 10 in Fig. 3 contain the same growth factor, a polypeptide with a M, of 16,400. In other purification experiments, the isoelectric point of the cartilage-derived growth factor varied from 9 to 10. Therefore, it seems that preparative isoelectric focusing does not permit an exact eval-

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**Cartilage-derived Growth Factor**

![Diagram](image.png)

**Fig. 4.** Estimation of polypeptide molecular weight by gel filtration chromatography and SDS polyacrylamide gel electrophoresis. Analysis of cartilage-derived growth factor activity recovered from the fraction with a pI of 9 in Fig. 3. *Left*, the pI 9 fraction was analyzed by gel filtration chromatography on Sephadex G-75 equilibrated with 4 M guanidine hydrochloride and 5 mM dithiothreitol. The 3-ml fractions were dialyzed against H2O and tested for their ability to stimulate the incorporation of [H]thymidine into 3T3 DNA. The molecular weight markers used were myoglobin (M, = 17,800), lysozyme (M, = 14,300), and insulin (M, = 5,800). *Right*, the active fractions from the Sephadex G-75 column were analyzed by SDS polyacrylamide gel electrophoresis. Slot 1 markers are phospholipase (M, = 94,000), albumin (M, = 68,000), ovalbumin (M, = 43,000), carbonic anhydrase (M, = 30,000), soybean trypsin inhibitor (M, = 21,000), and lysozyme (M, = 14,300). Slot 2, Fraction 16; slot 3, Fraction 17; slot 4, Fraction 18; and slot 5, Fraction 19. The arrow designates the polypeptide with a molecular weight of 16,400. B.D., blue dextran.

**Fig. 5.** Estimation of polypeptide molecular weight by gel filtration and SDS polyacrylamide gel electrophoresis. Analysis of cartilage-derived growth factor activity with a pI of 10 shown in Fig. 3. *Left*, the pI 10 fraction was analyzed by gel filtration chromatography on Sephadex G-75 equilibrated with 4 M guanidine hydrochloride and 5 mM dithiothreitol. *Right*, the active fractions of the Sephadex G-75 column were analyzed by SDS polyacrylamide gel electrophoresis. Slot 1, markers (see Fig. 4); slot 2, Fraction 18; slot 3, Fraction 20; slot 4, Fraction 21. The arrow designates the polypeptide with a molecular weight of 16,400. L designates the top of the lower gel. B.D., blue dextran.
cartilage-derived growth factor. Fraction synthesis in quiescent Balb/c 3T3 cells that is stimulated by analyzed by a repeated gel filtration chromatography on the concentration of about in Figs.

Recovery and Yield—The purification scheme is summarized in Table I. The yield of growth factor purified as outlined in Figs. 1, 3, 4, and 6 was about 60 µg starting with 1 g of crude extract. The recovery of growth factor activity was about 1%. DNA Synthesis and Cell Division—The amount of DNA synthesis in quiescent Balb/c 3T3 cells that is stimulated by increasing amounts of purified cartilage-derived growth factor is shown in Fig. 10. Half-maximal stimulation occurs at a concentration of about 2.5 µg/ml. Autoradiography of labeled nuclei (not shown) indicates that about 50% of the 3T3 nuclei are labeled at a concentration of 2.5 µg/ml, and 100% of the 3T3 nuclei are labeled at a concentration of 4 µg/ml. Purified cartilage-derived growth factor stimulates the proliferation of 3T3 cells as well as the synthesis of DNA. The number of 3T3 cells in a confluent monolayer increases from 8,600 to 24,800 after 4 days of incubation with purified growth factor at a concentration of 3 µg/ml.

Elution of Growth Factor After SDS-PAGE—Purified cartilage-derived growth factor was electrophoresed on SDS-PAGE. A portion of the polyacrylamide gel slab (Fig. 11, left) including molecular weight markers (slot 1) and purified cartilage-derived growth factor (slot 2, arrow) was fixed and stained. The rest of the polyacrylamide gel slab was not fixed. The section of the unstained gel (arrow) corresponding to the stained 16,400 M, polypeptide was excised and incubated overnight in 1.0% SDS in order to elute the growth factor. A portion of the eluate was analyzed by SDS-PAGE (Fig. 11, left, slot 3). The rest was analyzed by chromatography on Sephadex G-75 equilibrated with 4 M guanidine hydrochloride and 5 mM dithiothreitol (Fig. 11, center). Growth factor activity was found in the region corresponding to a molecular weight between 16,000 and 18,000 (Fractions 18, 19, and 20). The active fractions of the column were analyzed by SDS-PAGE (Fig. 11, right). A single polypeptide (arrow) with a molecular weight of 16,400 appeared in the most active column fraction, 19 (slot 7). It is apparent that the purified 16,400 M, polypeptide retains growth factor activity after electrophoresis on SDS-PAGE and gel elution. Elution of biologically active growth factor after SDS-PAGE of purified growth factor is further evidence that the 16,400 M, polypeptide and not some minor contaminant is the cartilage-derived growth factor.

Radioiodination—Purified cartilage-derived growth factor was labeled with 125I using the chloramine-T technique described under "Materials and Methods." The labeled growth

fig. 6. Purification of cartilage-derived growth factor by repeated gel filtration chromatography on Sephadex G-75. Fraction 17 of the Sephadex G-75 column described in Fig. 4 was analyzed by a repeated gel filtration chromatography on the same Sephadex G-75 column. Larger fractions (4.7 ml) were collected, dialyzed against distilled H2O, and tested for the ability to stimulate [3H]thymidine incorporation into 3T3 and chondrocyte DNA. O—O, DNA synthesis in 3T3 cells; ●—●, DNA synthesis in chondrocytes. B.D., blue dextran.

fig. 7. SDS-polyacrylamide gel electrophoresis of purified cartilage-derived growth factor. Fraction 11 of the Sephadex G-75 column described in Fig. 6 was analyzed by SDS polyacrylamide gel electrophoresis (slot 1 in the inset). The polypeptide distribution in this slot was monitored by densitometry. U, top of stacking gel; L, top of separating gel; B, bottom of gel. Slot 2 in the inset contains proteins of known molecular weight as described in Fig. 4.

fig. 8. Acid-urea polyacrylamide gel electrophoresis of purified cartilage-derived growth factor. Fraction 11 of the Sephadex G-75 column described in Fig. 6 was analyzed by polyacrylamide gel electrophoresis at low pH and in the presence of 6.25 M urea (slot 2 in the inset). The distribution of polypeptide in the slab gel was monitored by densitometry. T, top of gel; B, bottom of gel. The migration pattern of calf thymus histones (Worthington), H1, H2A, H2B, H3, and H4, is shown in slot 1.
factor was analyzed by SDS-PAGE (Fig. 9). Unlabeled cartilage-derived growth factor stained with Coomassie blue is shown in slot 2. Iodinated growth factor eluted from a Sephadex G-25 column with buffer containing albumin is analyzed in slot 3. The albumin (ALB) is detectable by Coomassie blue staining but the small amount of labeled growth factor (0.005 μg) is not. Slot 4 shows an autoradiogram of slot 3 after a 3-day exposure on x-ray film. The 16,400 M, polypeptide (arrow) is radiolabeled, and there is no evidence for the presence of minor contaminants.

**Amino Acid Analysis**—The amino acid composition of purified cartilage-derived growth factor is shown in Table II.

![Fig. 9](image-url)  
*Fig. 9. Radioiodination of cartilage-derived growth factor (CDGF).* Cartilage-derived growth factor was labeled with [125I]iodine and analyzed by autoradiography after SDS polyacrylamide gel electrophoresis. Slot 1, M, markers (see Fig. 4); slot 2, unlabeled cartilage-derived growth factor used for iodination (25 μg); slot 3, cartilage-derived growth factor labeled with [125I]iodine and separated from unreacted [125I]iodine by gel filtration on Sephadex G-25 equilibrated with 0.5% serum albumen (ALB) and 0.05 M phosphate, pH 7.5 (0.005 μg, 4 x 10^6 cpm). Slots 1, 2, and 3 are stained with Coomassie blue. Slot 4, autoradiography of sample in slot 3. The slab gel was dried and placed in contact with x-ray film for 3 days. The film was developed and photographed.

**Table I**

| Purification of the cartilage-derived growth factor from crude cartilage extract |
|-----------------------------------------------|
| Cartilage extract | Total protein | Units/# mg | % Recovery of activity |
| Cartilage extract | 1,000 | 18.5 | 18,500 |
| Bio-Gel A-0.5m | 54 | 97 | 5,240 | 28 |
| Isoelectric focusing | 2.88 | 294 | 847 | 4.5 |
| Sephadex G-75 | 0.21 | 1,250 | 263 | 1.4 |
| Sephadex G-75 | 0.06 | 3,030 | 182 | 1.0 |

*A A unit of activity is defined as the half-maximum obtainable incorporation of [3H]thymidine into the DNA of quiescent Balb/c 3T3 cells in a microtiter well. Under the standard conditions described under "Materials and Methods," the maximal incorporation in a typical microtiter well containing about 20,000 cells is between 80,000 and 100,000 cpm whereas background incorporation is between 2,000 and 4,000 cpm.

![DNA Synthesis Graph](image-url)  
*Fig. 10. Dose-dependent stimulation of DNA synthesis by cartilage-derived growth factor (CDGF).* DNA synthesis was measured in 3T3 cells after addition of varying concentrations of purified cartilage-derived growth factor.

**Table II**

| Amino acid composition of cartilage-derived growth factor |
|----------------------------------------------------------|
| The data represents the average of six hydrolyses performed on four separate samples. |
| Amino acid | Residues/mol |
|---------|--------------|
| Aspartic acid | 12.8 |
| Threonine | 6.9 |
| Serine | 1.8 |
| Glutamic acid | 17.2 |
| Proline | 5.3 |
| Alanine | 13.9 |
| Alanine | 13.1 |
| Valine | 7.5 |
| Methionine | 1.3 |
| Isoleucine | 4.7 |
| Leucine | 11.4 |
| Tyrosine | 2.4 |
| Phenyllalanine | 4.8 |
| Histidine | 6.4 |
| Lysine | 11.1 |
| Arginine | 3.6 |
| Half-cystine | 1.1 |
| Tryptophan | N.D. |

* Moles of amino acid per mol of cartilage-derived growth factor whose molecular weight is estimated to be 16,400.
* Based on extrapolation of data obtained by hydrolyses of 24, 48, and 72 h.
* Represents moles of cysteic acid generated by performic acid oxidation.
* Not determined.

The polypeptide is characterized by a low amount of half-cystine residues which is consistent with the resistance of growth factor activity to inactivation by sulfhydryl-reducing agents (1). The polypeptide also has relatively high amounts of lysine and histidine residues consistent with its cationic nature.

**Lysozyme and Trypsin Inhibitor Activity**—Two other proteins of low molecular weight have been demonstrated to be present in bovine scapular cartilage, lysozyme (17) and a protease inhibitor (10, 18). The cartilage-derived growth factor has no lysozyme activity and no trypsin inhibitor activity. In addition, neither egg white lysozyme nor cartilage-derived trypsin inhibitor stimulates DNA synthesis in Balb/c 3T3 cells. Lysozyme activity can be assayed by measuring degradation of Micrococcus luteus cells embedded in agar-
coated slides (17). Egg white lysozyme is active at concentrations as low as 0.05 μg/10 μl, while pure cartilage-derived growth factor has no lysozyme activity even at a concentration of 5 μg/10 μl. In addition, while the same growth factor preparation stimulated half-maximal DNA synthesis in Balb/c 3T3 cells at 1 μg/ml, egg white lysozyme had no stimulatory activity even at 50 μg/ml. The trypsin inhibitor activity can be assayed by measuring zones of inhibition on a slide containing fibrinogen embedded into agar and loaded with trypsin in a center trough (19). A partially purified trypsin inhibitor prepared by affinity chromatography on Sepharose-trypsin (10) inhibits trypsin activity at concentrations as low as 0.025 μg/10 μl. Purified cartilage-derived growth factor shows no inhibition of trypsin even at 2.5 μg/10 μl. The cartilage-derived trypsin inhibitor does not stimulate DNA synthesis in Balb/c 3T3 cells even at 100 μg/ml.

**DISCUSSION**

A growth factor with a molecular weight of 16,400 has been isolated from scapular cartilage and purified to homogeneity by a combination of gel filtration in the presence of 4 M guanidine hydrochloride and 5 mM dithiothreitol with 4 M guanidine hydrochloride and 5 mM dithiothreitol. The homogeneity of the purified cartilage-derived growth factor is ascertained by the presence of a single polypeptide band in two polyacrylamide gel electrophoresis systems, SDS-PAGE which separates polypeptides on the basis of their molecular weight and acid-urea PAGE which separates polypeptides mainly on the basis of their charge. In addition, if an apparently homogeneous preparation of growth factor is labeled with [35S]iodine, only one band, with a molecular weight of 16,400, is found in the autoradiogram of the radiolabeled material after SDS-PAGE. No minor bands of other molecular weight are detectable. The evidence that the 16,400 M₉ polypeptide is a growth factor is as follows. 1) Gel filtration in the presence of guanidine hydrochloride and dithiothreitol, a method used for the determination of polypeptide molecular weight (16), indicates that all of the cartilage-derived growth factor activity has a molecular weight between 16,000 and 18,000. 2) The relative distribution of growth factor activity in the active fractions of the gel filtration column correlates closely with the relative distribution of the 16,400 M₉ polypeptide on the slab gel after SDS-PAGE of the active column fractions. 3) Purified 16,400 M₉ polypeptide can be analyzed by SDS-PAGE and then eluted from the polyacrylamide slab after the electrophoresis is over. Gel filtration of the eluate yields growth factor activity with a molecular weight between 16,000 and 18,000, and a single polypeptide band of molecular weight 16,400 is found upon SDS-PAGE analysis of the active column fractions.

Amino acid analysis of the cartilage-derived growth factor indicates it to be different from other growth factors whose amino acid composition has been determined. These include epidermal growth factor (20), nerve growth factor (21), fibroblast growth factor (22), nonsuppressible insulin-like activity (23), somatomedin A (24), and somatomedin B (25). An important structural feature of the growth factor is that it contains at most one-half cystine residue per polypeptide. The low level of half-cystine residues is consistent with the resistance of the cartilage-derived growth factor to inactivation by sulfhydryl-reducing reagents (1). Growth factors found in the blood such as the platelet-derived growth factor (2), nonsuppressible insulin-like activity (3), multiplication-stimulating activity (4), and somatomedin C (5) are irreversibly inactivated by these reagents. Thus, disulfide bridges are important for maintaining biological activity of the blood-derived growth factors but not the cartilage-derived growth factor.

The cartilage-derived growth factor is a cationic polypeptide. However, the exact isoelectric point of this polypeptide is not certain. During the course of several purifications, the isoelectric point of the cartilage-derived growth factor, as determined by preparative isoelectric focusing on a column, has been found to be as low as pI 9 and as high as pI 10. In some experiments (Fig. 3), growth factor activity with both a pI of 9 and 10 have been observed on the same column. Purification of growth factor activity with a pI of 9 or 10 or a value between 9 and 10 always results in the preparation of a homogeneous and biologically active polypeptide with a molecular weight of 16,400. It is not clear what causes the variation in isoelectric point. Urea (6 M) has been introduced into the isoelectric focusing column to help promote dissociation of growth factor activity and to prevent the formation of precipitates. It is known that denaturation of a protein has an effect on its isoelectric point (11). For example, in the presence of 6 M urea, thyroglobulin is more heterogeneous than in the absence of 6 M urea, and the isoelectric point is increased by as much as 0.7 pH unit. The variations in the isoelectric point might reflect species of cartilage-derived growth factor in different states of denaturation.

Purification of the cartilage-derived growth factor involves...
extensive use of the denaturing reagent, guanidine hydrochloride. The guanidine hydrochloride is necessary for the extraction of cartilage and is used in the gel filtration steps to prevent aggregation. Gel filtration in the absence of guanidine hydrochloride results in a heterogeneous distribution of growth factor activity with molecular weights ranging from 25,000 to 150,000. Another denaturing reagent, urea, is used in the preparative isoelectric focusing steps. The use of denaturing agents may have an adverse effect on the biological activity of the growth factor. We are presently attempting to purify growth factor from cartilage without the use of denaturing reagents. Growth factor activity is found in chondrocytes prepared by collagenase digestion of bovine scapula cartilage (6). The cellular growth factor activity is found to be totally associated with chromatin (6). If the chromatin is incubated with 0.7 M NaCl, growth factor activity with a molecular weight between 16,000 and 18,000 and which is resistant to inactivation by sulfhydryl-reducing reagents is released. The partially purified chromatin-derived growth factor stimulates DNA synthesis at concentrations of 0.5 to 1 μg/ml as compared to the pure cartilage-derived growth factor which is active at 1 to 2 μg/ml. Purification of growth factor using chromatin as the starting material rather than cartilage may eventually yield a preparation as active as epidermal growth factor (26), fibroblast growth factor (27), and platelet-derived growth factor (28). These growth factors stimulate DNA synthesis in 3T3 cells at a concentration of 1 to 10 ng/ml.

The cartilage-derived growth factor has another biological activity besides stimulating DNA synthesis in 3T3 cells and in chondrocytes. Capillary endothelial cells have been established in cell culture by Folkman et al. (29). The cartilage-derived growth factor stimulates the migration and proliferation of capillary endothelial cells at a concentration of about 0.5 μg/ml (31). The stimulation of capillary endothelial cell migration and proliferation by cartilage-derived growth factor reflects some specificity since epidermal growth factor which is a potent mitogen for 3T3 cells (26) has no stimulatory effect on the capillary endothelial cells. The physiological role of the cartilage-derived growth factor is speculative. Scapular cartilage is a developing tissue. The initial phase of cartilage development is characterized by rapid proliferation of chondrocytes. Eventually, the cartilage is invaded by capillaries, is destroyed, and replaced by bone. The factors that are involved in the growth and vascularization of cartilage in vivo have not been ascertained. The cartilage-derived growth factor might be an endogenous polypeptide whose role is to stimulate the growth of chondrocytes and blood vessels in a developmental sequence.

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