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Characterization of Growth Factors in Human Cartilage

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Growth factor activity has been identified in the chondrocytes and extracellular matrix (ECM) fractions of human costal cartilage. There was about five times more growth factor activity in the ECM than was found to be associated with the chondrocytes. The growth factor activity in chondrocytes was found to be associated with chromatin. Both the chromatin-associated growth factor (CAGF) activity and extracellular matrix growth factor (EMGF) activity were characterized for molecular weight, charge, and the effect of reduction by sulfhydryl reducing reagents. Biorex cation exchange chromatography showed that both CAGF and EMGF were cationic. CAGF and EMGF have molecular weights between 15,000 and 18,000 as determined by size exclusion chromatography on HPLC TSK 3000 columns equilibrated with guanidine-HCl and dithiothreitol.

Key words: chrondocytes, chromatin, human cartilage, extracellular matrix, growth factors

The growth of cartilage is an important component of normal skeletal development. A number of growth defects in humans involve abnormalities in the development, differentiation, and/or growth of cartilage. For example, in achondroplasia the dwarf-life stature observed is the result of the lack of cartilage growth. Both benign (chondroma) and malignant (chondrosarcoma) tumors arise from uncontrolled proliferation of cartilage cells. Arthritis results when cartilage degenerates in the absence of a normal repair mechanism.

The macromolecular factors that are involved in normal and abnormal human cartilage growth have not been well defined. A group of polypeptides found in human blood, known collectively as the somatomedins or insulinlike growth factors (IGF), are thought to mediate the growth promoting effects of growth hormone [1,2]. Thus, the failure of Laron-type dwarfs to respond to growth hormone is associated with low somatomedin levels [1,2]. In a recent report Schoenle et al [3] present data indicating that administration of pure human IGF-I to hypophysectomized rats stimulates an increase in tibial epiphyseal width, an increase in DNA synthesis in vitro in costal cartilage, and a gain in body weight. These effects are similar to those observed following administration of human growth hormone to hypophysectomized rats [3].

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In addition to the somatomedins, there are several other human factors that influence chondrocyte function in vitro. Two polypeptides have been identified in conditioned media from cultures of human blood mononuclear cells. A lymphokine, described by Herman et al [4], induces a concentration-dependent, reversible suppression of chondrocyte glycosaminoglycan (GAG) and protein synthesis. Jasin and Dingle [5] have identified a factor produced by adherent mononuclear cells which induces the degradation of matrix proteolycan and collagen in cartilage explants in organ culture. These factors could play a role in cartilage destruction associated with degenerative joint disease. In addition, human synovial cells produce a factor, or factors, which stimulate secretion of plasminogen activator by chondrocytes [6] and stimulate chondrocyte-mediated degradation of cartilage matrix [7]. The structural relationships of these factors have not been determined.

Our laboratory has previously isolated growth factors from bovine articular and scapular cartilage which stimulate DNA synthesis and cell division in 3T3 cells and in chondrocytes in vitro [8,9]. At present two growth factors have been identified. One factor, referred to as the cartilage-derived growth factor (CDGF), has been purified to homogeneity from a 1 M guanidine extract of whole bovine scapular cartilage [8]. CDGF is a cationic polypeptide with a molecular weight of 16,400. A second growth factor has been isolated in association with the chromatin fraction of bovine scapular and articular chondrocytes [9]. The chromatin-associated growth factor (CAGF) is cationic and has a molecular weight between 20,000 and 22,000. The structural and functional relationships between CDGF and CAGF have not been defined.

One of our goals is to study the role of growth factors in both the normal and abnormal development of human cartilage. In these studies we demonstrate that growth factor activity is present in human costal cartilage. Furthermore, when chondrocytes are separated from the extracellular matrix (ECM) components, both the cells and ECM are found to contain growth factor activity. The distribution of growth factor activity in the two cartilage fractions and the biochemical properties of the human cartilage-derived growth factors are described.

MATERIALS AND METHODS

Preparation of Chondrocytes and Extracellular Matrix

Human costal cartilage was obtained from the Surgical Service at Children's Hospital Medical Center. The cartilage was finely diced and resuspended in Dulbecco's phosphate-buffered saline (PBS), pH 7.5, containing 0.2% clostridial collagenase (150 units/mg, Worthington CLS II), penicillin (200 units/ml), and streptomycin (200 μg/ml). The cartilage was digested in a capped sterile tube for 16–18 hr at 37°C with constant agitation. The digest was passed through a 153-μm nylon sieve (Tetko, HC3-110, Elmsford, New York) to remove debris and undigested material. Chondrocytes were sedimented by centrifugation at 1,000 g for 10 min. The supernatant, which is designated as the extracellular matrix, was removed and stored in a separate tube. The chondrocytes were then washed three times in PBS supplemented with antibiotics. The cells were greater than 80% viable as determined by trypan blue exclusion.

Measurement of growth factor activity in chondrocytes and ECM was preceded by sonication of the two fractions with a Branson Sonifier Cell Disruptor at 50 W for two pulses for 15 sec. The samples were centrifuged at 1,000 g for 15 min, filtered

through an 0.45-μm Nalgene filter, dialyzed (spectrophor tubing 6,000–8,000 molecular weight cutoff, Fisher Scientific Co., Medford, Massachusetts) at 4°C for 48 hours vs three changes of deionized, distilled (dd) water.

Preparation of Chromatin-Associated Growth Factor Activity

The preparation of chromatin has been described previously [9]. Growth factor activity was extracted from the human chondrocyte chromatin with NaCl. An aliquot of chromatin was resuspended in TEP buffer (10 mM Tris-HCl, 0.2 mM sodium EDTA, and 0.1 mM phenylmethyl fluoride, pH 8.0) supplemented with 0.1 M NaCl. The chromatin was centrifuged at 14,000g for 10 min. The supernatant fraction was discarded and the pellet was resuspended in 10 ml of TEP buffer supplemented with 1.0 M Nacl. The suspension was layered onto a sucrose shelf containing 15 ml of 20% sucrose in TEP buffer supplemented with 1.0 M NaCl. After centrifugation for 1 hr at 100,000g in a Spinco Ti 60 rotor (Beckman, Palo Alto, California), the 10-ml layer above the sucrose shelf, which contained the extracted protein, was removed with a pipette, and dialyzed against dd H_2O. This fraction is referred to as the chromatin salt extract.

Measurement of DNA Synthesis in Cell Culture

Growth factor activity was assayed by measuring stimulation of DNA synthesis and cell division in confluent monolayers of quiescent mouse Balb/c 3T3 cells and/or primary cultures of human chondrocytes. The preparation of quiescent monolayers of 3T3 cells and the methods used to measure DNA synthesis and cell division in these cells have been previously described [10]. The same methods were used to measure DNA synthesis in human costal cartilage chondrocytes except that quiescence was produced by changing the serum concentration from 10% to 1% 3 days after plating, and waiting 7 more days prior to testing for growth factor activity. The amount of growth factor in 250 μl that is required to stimulate half-maximal synthesis in quiescent 3T3 cells is defined as a unit of activity. In a typical microtiter well, containing about 20,000 3T3 cells, the background stimulation is about 5,000 cpm and maximal stimulation is 100,000–120,000 cpm.

High-Performance Liquid Chromatography (HPLC)

Cartilage-derived growth factor activity was analyzed on a Beckman Model 334 HPLC gradient system using a TSK 3000 size exclusion column (7.5 mm ID × 50 cm, Varian) equilibrated with 6 M guanidine-HCl, 5 mM dithiothreitol (DTT), and 0.02 M 2-(morpholino)ethane sulfonic acid (MES), pH 6.5. HPLC was carried out at room temperature at a flow rate of 1.0 ml/min. Fractions of 0.85 ml were collected, dialyzed against dd water, and tested for the ability to stimulate DNA synthesis in 3T3 cells and/or human chondrocytes. The columns were calibrated with the following molecular weight standards: blue dextran (MW 2×10^6), ovalbumin (MW 43,000), myoglobin (MW 17,800), ribonuclease (MW 13,700), and insulin (MW 5,800, subunit MWs of 3,100 and 2,400).

Cation Exchange Chromatography

Biorex 70 cation exchange resin was equilibrated in 0.01 M Tris-HCl/0.1 M NaCl/1 mM PMSF, pH 8.0, buffer at 4°C. Samples of cartilage growth factor were lyophilized and resuspended in the equilibration buffer and applied to the column at a

flow rate of 24 ml/hr. After rinsing the column with three column volumes of equilibration buffer, the bound material was eluted with a 70 ml NaCl gradient (0.1–1.0 M) in 0.01 M Tris, pH 8. Additional material which remained bound after exposure to 1.0 M salt was eluted with 3.0 M NaCl. Fractions of 4 ml were collected and tested for their ability to stimulate DNA synthesis in cultured cells. The salt concentration was determined by measuring the conductivity (expressed in millisohms) of each fraction on a conductivity meter (Radiometer, Rainin Instrument Co). A standard curve was constructed using salt solutions of known concentration.

Assessment of Stability of Growth Factor Activity

A defined amount of growth factor activity from sonicated cells and ECM was exposed to the following: 6 M guanidine-HCl, pH 6.5; 6 M guanidine-HCl; 5 mM DTT, pH 6.5; and 5 mM DTT, pH 6.5. The samples were incubated for 1 hr at room temperature. The material was then dialyzed exhaustively against dd water, lyophilized, and reconstituted in water for analysis of growth factor activities.

RESULTS

Identification of Growth Factor Activity in Chondrocytes and ECM Obtained From Human Costal Cartilage

Chondrocytes can be separated from the ECM of cartilage by digestion with collagenase. In order to determine whether human costal cartilage contained growth factor activity, sonicates of chondrocytes, and ECM were added to confluent, quiescent Balb/c 3T3 cells in the presence of (methyl-^{3}H) thymidine (Fig. 1). Both the chondrocyte and ECM-derived material stimulated DNA synthesis in the cultured cells in a dose-dependent manner. There is approximately five times more growth

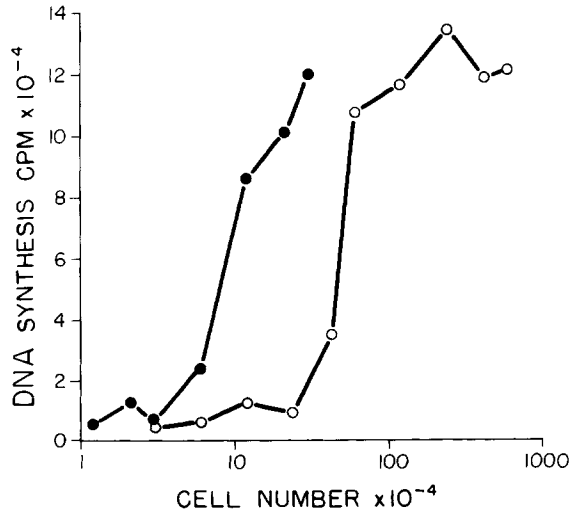

Fig. 1. Growth factor activity in extracellular matrix (ECM) and chondrocytes obtained from human costal cartilage. Chondrocytes and ECM isolated from a given number of chondrocytes were sonicated and analyzed for their ability to stimulate DNA synthesis in 3T3 cells. (●) ECM: (○) cell.

factor activity in the ECM than in the chondrocytes. Whereas the ECM prepared from about 10^4 chondrocytes is required for 50% maximal stimulation, 5×10^4 chondrocytes are required for similar activity.

Determination of the Molecular Weights of the Cellular and ECM-Derived Growth Factor Activities

The molecular weight of polypeptide subunits can be determined by size exclusion chromatography in the presence of guanidine and DTT [11]. Cellular and ECM growth factor activity was analyzed on HPLC TSK 3000 size exclusion columns equilibrated with 6 M guanidine-HCl and 5 mM DTT (Fig. 2). The growth factor activity derived from the chondrocytes and the ECM both have molecular weights in the range of 15,000–18,000. The peaks of cellular and ECM-induced activity seem to overlap, but since some cell-derived growth factor activity is observed at a slightly greater molecular weight than the ECM-derived material, it is possible that the former is a little larger than the latter.

It was found that the supplementation of guanidine-HCl with DTT is required for the maintenance of growth factor activity. Both the cellular and ECM-derived growth factor preparations fail to stimulate DNA synthesis following exposure to 6 M guanidine-HCl alone. However, growth factor activity is retained following exposure to either 6 M guanidine-HCl supplemented with 5 mM DTT or 5 mM DTT alone (Table I). Furthermore, the peak of activity found after HPLC on TSK 3000 in the presence of guanidine-HCl and DTT was not found after HPLC in guanidine-HCl alone (results not shown).

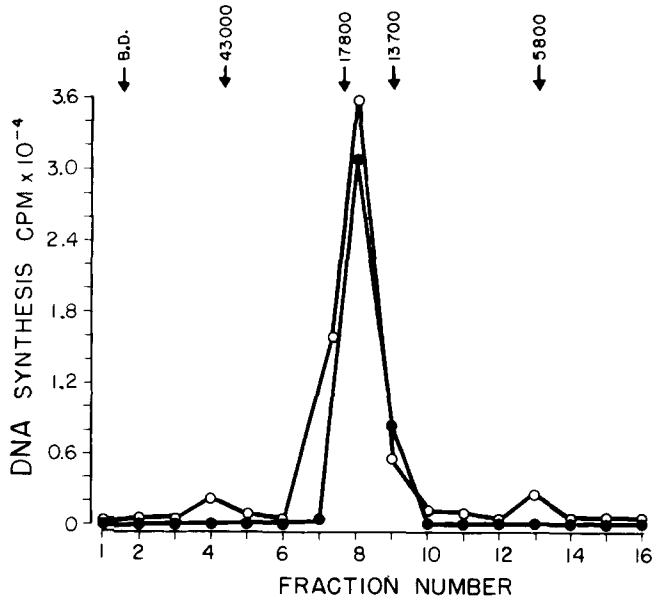

Fig. 2. Molecular weight analysis of growth factor activity by HPLC. About 360 units of ECM material and 350 units of cellular material were applied to a TSK 3000 size exclusion column equilibrated with 6 M guanidine-HCl and 5 mM DTT pH 6.5. Individual fractions were dialyzed against dd water and analyzed for their ability to stimulate DNA synthesis in 3T3 cells. (●) ECM; (○) cell.

TABLE I. Effects of Guanidine-HCl and Dithiothreitol on Growth Factor Activity*

Exposure to	DNA synthesis (cpm) EMGF	CAGF
H_2O	150,000	50,000
6 M guanidine-HCl	3,000	1,000
5 mM dithiothreitol	92,000	48,000
6 M guanidine-HCl plus 5 mM dithiothreitol	110,000	66,000

*Aliquots of EMGF and CAGF were exposed to H_2O, guanidine-HCl, and dithiothreitol for 1 hr, dialyzed against H_2O, and tested for the ability to stimulate DNA synthesis in quiescent 3T3 cells.

Analysis of Growth Factor Activity Following Cation Exchange Chromatography

Previous work with bovine chondrocytes indicated that all of the cellular growth factor activity is associated with chromatin [9]. Analysis of human costal chondrocytes demonstrated that the growth factor activity was also associated with chromatin (results not shown). The association of growth factor activity with chromatin suggests that the growth factor is cationic. In these studies both the ECM-derived and chromatin-associated growth factors were analyzed by ion exchange chromatography on Biorex 70 cation exchange resin (Fig. 3A, B). All of the growth-promoting activity from both sources is retained by the column. Approximately 0.4 M (Fig. 3A) and 0.5 M (Fig. 3B) NaCl is required for elution of the ECM-derived and chromatin-associated growth factors, respectively. Thus, both growth factors appear to have a net positive charge. Similar results were found using either quiescent 3T3 cells (Fig. 3) or human chondrocytes (data not shown) to measure growth factor activity.

DISCUSSION

Human costal cartilage contains growth factors that stimulate DNA synthesis in 3T3 cells and in human chondrocytes. Growth factor activity can be found in both the extracellular matrix fraction of collagenase-digested cartilage and in association with the chromatin fraction of the chondrocyte. The cartilage extracellular matrix growth factor (EMGF) is approximately five times as potent as the chromatin-associated growth factor (CAGF) when expressed on a per cell basis. However, it is unclear whether this represents the relative amount of growth factor present in the ECM versus the chondrocyte or a difference in the potency of the two factors. The relationship between EMGF and CAGF is unclear. The factors have some structural similarities in that both are cationic and have similar molecular weights in the range of 15,000–18,000. In addition, EMGF and CAGF activity is resistant to inactivation by the sulfhydryl reducing reagent DTT.

The cartilage-derived growth factors have some interesting physical properties. Unlike other growth factors such as platelet-derived growth factor [12] and somatomedin C [13], EMGF and CAGF are resistant to irreversible inactivation by sulfhydryl reducing reagents. Moreover, under certain conditions such as denaturation with guanidine-HCl, sulfhydryl reducing reagents such as DTT are actually required to

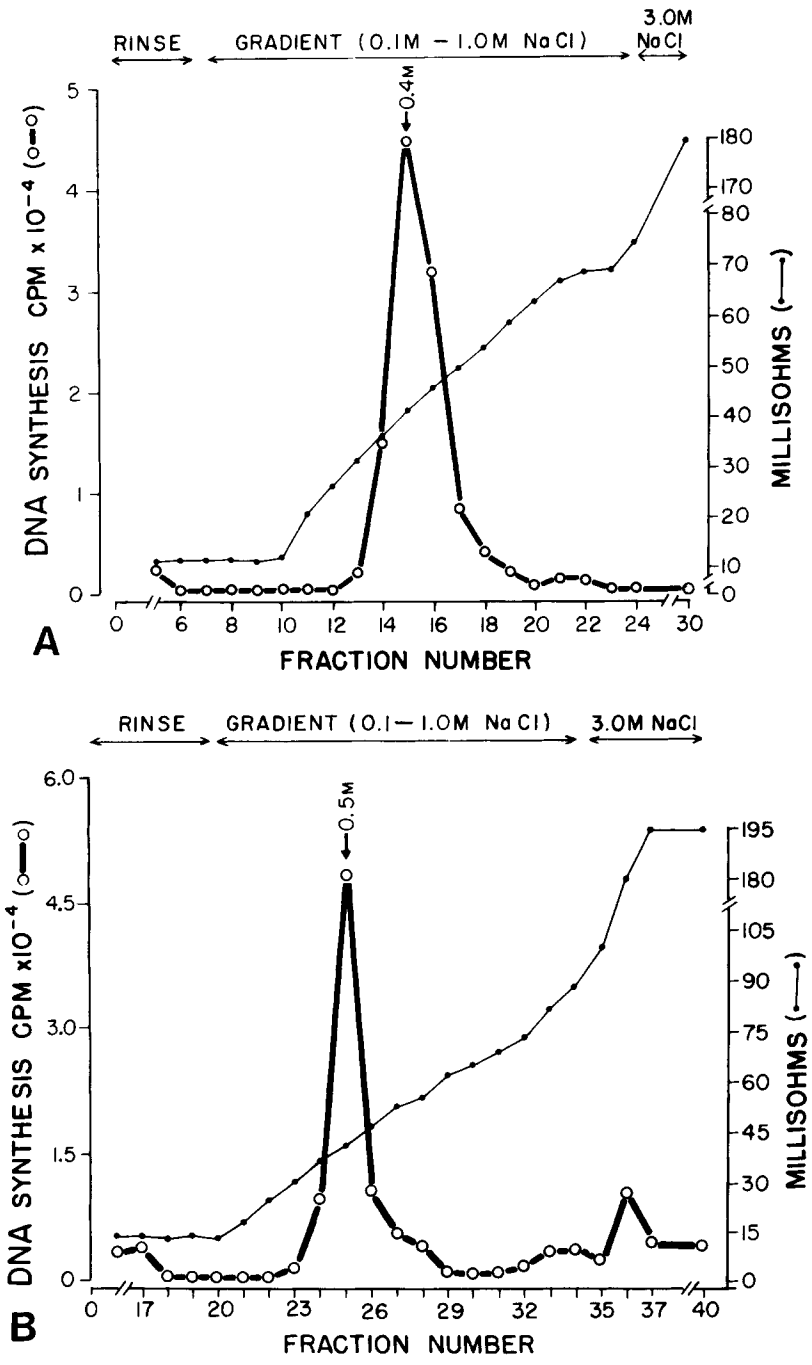

Fig. 3. Biorex 70 cation exchange chromatography. Growth factor activity was applied to a Biorex 70 (0.9 × 7.5 cm) ion exchange column and eluted with a gradient (0.1–1.0 M) of NaCl. The salt concentration required for elution (arrow) of growth factor activity was calculated by measuring the conductivity (millisohms) of each fraction and comparing values to a calibrated curve of millisohms and NaCl concentration. A) About 66 units of ECM; B) about 500 units of CAGF.

maintain biological activity. The structural basis for the protective function of DTT is unclear. It is possible that the formation of disulfide bridges between sulfhydryl groups exposed by denaturation with guanidine-HCl leads to inactivation of the growth factors.

EMGF and CAGF appear to be endogenous to cartilage. Their structural characteristics indicate that they are probably different from other exogenous human factors known to stimulate chondrocyte growth and function. These exogenous factors include a group of blood-derived polypeptides collectively referred to as somatomedins [1,2,14], a chondrocyte growth factor (CGF) isolated from the human pituitary gland [15], a lymphokine which induces a reversible suppression of glycosaminoglycan and protein synthesis by chondrocytes [4], and a factor(s) produced by adherent mononuclear cells that has been demonstrated to stimulate chondrocytes in organ culture to degrade matrix macromolecules [5]. The cartilage-derived growth factors can be distinguished from the somatomedins, CGF, and lymphokine on the basis of their physical characteristics. EMGF and CAGF differ substantially from these other factors in molecular weight. The somatomedins have a molecular weight of 7,000–8,000 [14], CGF has a molecular weight of approximately 40,000 [15], and the lymphokine has a molecular weight of approximately 53,000 [4]. Furthermore, EMGF and CAGF activity is maintained following exposure to sulfhydryl reducing reagents. In contrast, the somatomedins lose activity following reduction [14]. The effect of reducing agents on CGF and the lymphokine has not been reported. The relationship between the cartilage factors and the matrix-degrading activity (MDA) produced by adherent mononuclear cells is less clear since MDA has not been extensively characterized. The molecular weight of MDA is reported to be between 12,000 and 20,000 [5]. Further comparisons await additional biochemical characterization of MDA and the analysis of additional functional properties of the cartilage-derived factors.

Since the cartilage-derived growth factors stimulate DNA synthesis in 3T3 cells, it is appropriate to compare these factors to other growth factors that are known to stimulate 3T3 cells. The best characterized of these growth factors are epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF). The cartilage-derived factors are clearly different from EGF and PDGF. EGF is a polypeptide with a molecular weight of 6,200 and an isoelectric point of 4.7 [16]. PDGF has a molecular weight of 35,000 and an isoelectric point of about 9.7 [12]. Furthermore, PDGF is inactivated by exposure to sulfhydryl reducing agents. In contrast, the growth factors found in cartilage most closely resemble bovine FGF. FGF, which is found in bovine pituitary, is a cationic polypeptide with a molecular weight of about 13,000 and is resistant to inactivation by sulfhydryl reducing agents [17]. Furthermore, FGF has been shown to stimulate the proliferation of chondrocytes [17]. A structural comparison of pure cartilage-derived growth factor and FGF will be necessary to ascertain whether there is a relationship between these polypeptides.

The physiological role of the EMGF and CAGF has not been determined. In addition to stimulating chondrocyte growth, the bovine cartilage-derived growth factors have been shown to be potent mitogens for capillary endothelial cells [18]. It is possible that cartilage-derived growth factors stimulate chondrocyte growth in the avascular stage of cartilage development and blood vessel growth in the vascular stage which precedes bone formation. The presence of a growth factor in association with chromatin is intriguing. The CAGF may be involved in the activation of specific

genes required for cartilage development. Alternatively, the association with chromatin might represent a mechanism for sequestering the growth factor in the nucleus. Thus, a programmed release of CAGF may occur at a specific time during cartilage development. It is anticipated that further characterization of the structure and function of these macromolecular factors contributing to normal cartilage growth and development will elucidate the mechanisms responsible for the aberrant growth observed in diseases affecting cartilage and bone.

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REFERENCES

1. Phillips LS, Vassilopoulou-Sellin R: N Engl J Med 302:371, 1980.
2. Posner BI, Guyda HJ: In Bing, DH, Rosenbaum RA (eds): "Plasma and Cellular Modulatory Proteins." Boston: Center for Blood Research, 1981, p 15.
3. Schoenle E, Zapf J, Humbel RE, Froesch ER: Nature 296:252, 1982.
4. Herman JH, Nutman TB, Nozoe M, Mowery CS, Dennis MV: Arthritis Rheum 24:824, 1981.
5. Jasin HE, Dingle JT: J Clin Invest 68:571, 1981.
6. Meats JE, McGuire MB, Russell RGG: Nature 286:891, 1980.
7. Dingle JT, Saklatvala J, Hembry R, Tyler J, Fell HB, Jubb R: Biochem J 184:177, 1979.
8. Klagsbrun M, Smith S: J Biol Chem 255:10859, 1980.
9. Azizkhan JC, Klagsbrun M: PNAS 77:2762, 1980.
10. Klagsbrun M, Langer R, Levenson R, Smith S, Lillehei C: Exp Cell Res 105:99, 1977.
11. Fish WW, Mann KG, Tanford C: J Biol Chem 244:4989, 1969.
12. Antoniades HN, Scher CD, Stiles CD: Proc Natl Acad Sci USA 76:1809, 1979.
13. Van Wyk JJ, Furlanetto RW, Plet AS, D'ercole AJ, Underwood LE: In Bailer JC III (ed): "National Cancer Institute Monograph 48." Bethesda, Maryland: National Cancer Institute, 1978, p 141.
14. Van Wyk JJ, Underwood LE: In Litwack G (ed): "Biochemical Actions of Hormones," Vol V. New York: Academic Press, 1978, p 101.
15. Kasper S, Worsley IG, Rowe JM, Shiu RPC, Friesen HG: J Biol Chem 257:5226, 1982.
16. Cohen S: J Biol Chem 237:1555, 1962.
17. Gospodarowicz D, Moran JS: In Snell EE, Boyer PD, Meister A, Richardson CC (eds): "Annual Review of Biochemistry." Palo Alto, California: Annual Reviews, Inc, 1976, p 531.
18. Zetter BR, Azizkhan RG, Azizkhan JC, Brouty-Boye D, Folkman J, Haudenschild CC, Klagsbrun M, Potash R, Scheiner CJ: In Bind DH, Rosenbaum RA (eds): "Plasma and Cellular Modulatory Proteins." Boston: Center for Blood Research, 1981, p 59.