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Purification of Two Distinct Growth Factors from Bovine Neural Tissue by Heparin Affinity Chromatography†

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ABSTRACT: Two growth factors have been purified to homogeneity from either bovine hypothalamus or brain by heparin affinity chromatography. Both stimulate the growth of murine 3T3 fibroblasts and bovine capillary endothelial cells. One heparin-binding growth factor (HGFα), purified from either tissue by elution from heparin with 1.0 M sodium chloride, is obtained in a yield of 0.4 mg/kg of tissue. Its apparent molecular weight is 16 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and its amino acid composition is identical with that of the acidic fibroblast growth factor recently isolated from bovine brain by a multistep chromatographic procedure [Thomas, K. A., Rios-Candelore, M., & Fitzpatrick, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 357–361]. A second growth factor (HGFβ), isolated from either tissue by elution from heparin with 2.0 M sodium chloride, is obtained in a yield of 0.02 mg/kg of tissue. Its apparent molecular weight is 18 000 by SDS–PAGE, and its amino acid composition differs from that of HGFα. These results confirm the existence of two distinct growth factors in bovine neural tissue and establish that the acidic endothelial cell growth factor from hypothalamus and the acidic brain fibroblast growth factor are identical.

Heparin affinity chromatography provides a powerful method for the isolation of endothelial cell growth factors (Shing et al., 1984; Klagsbrun & Shing, 1984a,b; D'Amore & Klagsbrun, 1984). A potent mitogen for capillary endothelium has been purified to homogeneity by this means from a rat chondrosarcoma (Shing et al., 1984) and from bovine cartilage (Sullivan & Klagsbrun, 1984). Moreover, the growth factors present in bovine neural tissue, including hypothalamus (Maciag et al., 1979, 1982), brain (Gospodarowicz et al., 1978, 1982), pituitary (Gospodarowicz, 1975), and retina (D'Amore et al., 1981), are adsorbed completely to immobilized heparin columns (Klagsbrun & Shing, 1984a,b; D'Amore & Klagsbrun, 1984). Large-scale extractions of bovine brain and hypothalamus have been performed according to the procedures of Gospodarowicz et al. (1978, 1982), coupled with heparin affinity chromatography to extend these results. This report describes the purification to homogeneity of the two heparin-binding growth factors present in both tissues.

Experimental Procedures

Materials. Bovine brain and hypothalamus were obtained from Pel-Freez Biologicals (Rogers, AR). CM-Sephadex C50 and heparin–Sepharose were from Pharmacia (Piscataway, NJ). Protein assay and silver nitrate stain kits were from Bio-Rad (Richmond, CA). Porcine heparin was from Sigma (St. Louis, MO). ECGS[1] was from Collaborative Research Inc. (Lexington, MA). [^{125}I]Iododeoxyuridine was from New England Nuclear (Boston, MA).

Cells. Bovine capillary endothelial (BCE) cells, clone 76-23, were the generous gift of Dr. Judah Folkman. Balb/c 3T3 cells, clone A31, subclone MK25, were the generous gift of Dr. Michael Klagsbrun. 3T3 cells were maintained at 37 °C in DME supplemented with 10% heat-inactivated bovine serum (M. A. Bioproducts, Walkersville, MD), L-glutamine (2 mM), gentamicin (50 μg/L), and fungizone (500 μg/L) (DME/10) in Costar flasks in a humidified 5% CO_2 in air incubator. BCE cells were maintained at 37 °C in the same medium supplemented with 90 μg/mL heparin and 20 μg/mL ECGS

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[1] Abbreviations: ECGS, endothelial cell growth supplement; ECGF, endothelial cell growth factor; HGF, heparin-binding growth factor; RDGF, retina-derived growth factor; FGF, fibroblast growth factor; BCE, bovine capillary endothelial; DME, Dulbecco's modified Eagle's medium; PBS, Dulbecco's calcium- and magnesium-free phosphate-buffered saline; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IdU, iododeoxyuridine; TFA, trifluoroacetic acid.

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(DME/10/HE; Thornton et al., 1983) on gelatin-coated Petri dishes (Folkman et al., 1979) in a humidified 10% CO_2 in air incubator.

Growth Factor Isolation. One-kilogram lots of bovine brain or hypothalamus were homogenized, acidified to pH 4.5, and processed with ammonium sulfate exactly as described (Gospodarowicz et al., 1982). The ammonium sulfate pellet was redissolved in water, dialyzed extensively against water in 6000–8000 molecular weight cutoff dialysis tubing (Spectrapor, Spectrum Medical Industries, Queens, NY), and finally dialyzed overnight at 4 °C against 2 L of 0.1 M sodium phosphate, pH 6.0. A large precipitate was removed by centrifugation (20000*g*, 20 min), and the supernatant was applied to CM-Sephadex C50 equilibrated in the same buffer at a flow rate of 80 mL/h. The column was washed with the same buffer until the absorption at 280 nm returned to its base line value, giving an unbound fraction (CMF1), and then eluted sequentially with the same buffer containing 0.15 and 0.5 M NaCl, giving fractions CMF2 and CMF3, respectively. Fraction CMF3 was dialyzed overnight against 10 mM Tris-HCl, pH 7.0, containing 0.6 M NaCl and applied directly at a flow rate of 25 mL/h to heparin–Sepharose (10-mL bed volume, 1.6 × 5 cm) equilibrated with the same buffer at 4 °C (Shing et al., 1984). The column was washed sequentially at the same flow rate with 10 mM Tris-HCl, pH 7.0, supplemented with 0.6 M NaCl, 1.0 M NaCl, and finally 2.0 M NaCl.

High-Performance Liquid Chromatography. Reversed-phase HPLC was performed by employing a Waters Associates liquid chromatography system consisting of an LKB 2138 206-nm detector, two model 6000 solvent delivery systems, and a propylsilane (C3) column (10-μm particle size, 250 × 4.1 mm; Synchrom Inc., Linden, IN). Elution at 23 °C was performed at a flow rate of 0.8 mL/min. Growth factor pools from the heparin column were applied directly to the C3 column through the pump and washed with 0.1% (v/v) TFA in water until absorption returned to base line. The column was eluted over 30 min with a linear gradient from 0% to 60% acetonitrile/0.1% TFA in water. One-minute fractions were collected in siliconized tubes.

Assays. Growth stimulation of quiescent, confluent monolayers of 3T3 cells was measured in 96 well plates by standard techniques exactly as described elsewhere (Klagsbrun et al., 1977), except that [^{125}I]iododeoxyuridine (IdU, 10 μCi/plate) was substituted for [^{3}H]thymidine (Marquardt et al., 1984).

Growth stimulation of BCE cells was assessed as follows: BCE cells were plated into gelatin-coated 96 well half-area plates (Costar) at 2500 cells per well in 100 μL of DME/10/HE. They were grown almost to confluence and then placed in DME/10 for 5–7 days. Thereafter, samples were loaded and plates processed as for 3T3 plates. For 3T3 stimulation, 20% bovine serum was used as a positive control, resulting in a 15–30-fold increase in IdU uptake. For BCE stimulation, serial dilutions of ECGS were used as positive controls, resulting in a small but highly reproducible 2–3-fold stimulation of IdU uptake at optimal levels (5–20 μg/mL). One unit of activity in either assay is defined as that amount of mitogen required to stimulate half-maximal IdU incorporation. All assays were performed in duplicate.

Aliquots from heparin and C3 column fractions were taken immediately upon completion of the column run and diluted 40–500-fold into PBS containing 0.1% bovine serum albumin. When necessary, fractions were diluted further into the same buffer until optimal levels were obtained for use in stimulation assays.

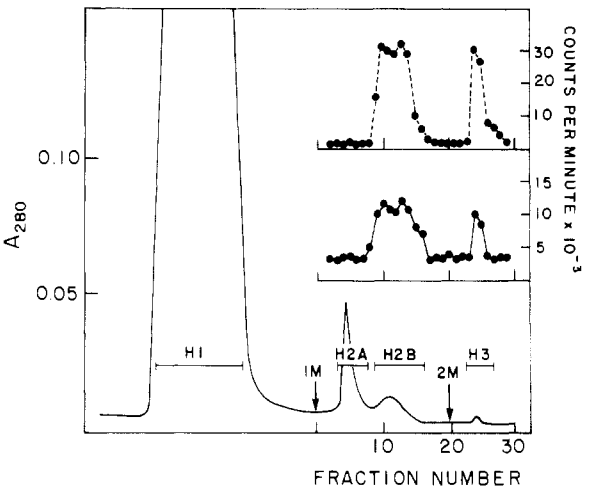

FIGURE 1: Chromatography of fraction CMF3 from 1 kg of bovine brain on heparin–Sepharose equilibrated with 10 mM Tris-HCl, pH 7.0, containing 0.6 M NaCl. The solid line indicates the absorbance change at 280 nm. The arrows identify the points of addition of elution buffers containing 1 and 2 M NaCl. Stimulation of IdU incorporation into the DNA of quiescent 3T3 cells (-●-) and BCE cells (–●–) by the fractions eluted with 1 and 2 M NaCl is also indicated.

Other Techniques. Analytical SDS–PAGE was performed on vertical slab gels according to standard techniques (Laemmli, 1970) in the absence of thiol reducing agents. Gels were silver stained by using a commercially available kit (Bio-Rad). Protein concentrations of crude fractions were determined by the dye binding assay of Bradford (1976). Protein concentrations of pure mitogens eluted from heparin or C3 columns were determined by amino acid analysis, which was performed by the "Pico-Tag" method (Waters Associates; Biddlingmeyer et al., 1984).

Results

(A) Optimization of Heparin–Sepharose Elution Conditions. Elution of heparin columns with salt gradients (Klagsbrun & Shing, 1984b; D'Amore & Klagsbrun, 1984) has established that the heparin-binding growth factors (HGF's) present in bovine neural tissue elute either at about 1.0 M NaCl or at about 1.5 M NaCl. Moreover, mitogens are retained on the column when applied at NaCl concentrations as high as 0.6 M (Shing et al., 1984). To obviate the need to perform gradients for large-scale preparations, conditions were optimized first to elute both mitogens sequentially at different constant salt concentrations. Small aliquots of partially purified fraction CMF3 from bovine brain were dialyzed against 10 mM Tris-HCl, pH 7.0, containing 0.6 M NaCl and applied to heparin–Sepharose, and contaminating proteins were eluted with the same buffer. The first heparin-binding growth factor (HGFα) was eluted with 10 mM Tris-HCl, pH 7.0, containing different concentrations of NaCl varying from 0.95 to 1.1 M (data not shown). An optimal separation of mitogen from contaminants was obtained at 1.0 M NaCl (see below). The second heparin-binding growth factor (HGFβ) was eluted subsequently with 2.0 M NaCl (see below).

(B) Purification of Mitogenic Activity from Bovine Brain and Hypothalamus. The elution profiles on heparin–Sepharose of fraction CMF3 partially purified from 1-kg extracts of either hypothalamus or brain are virtually identical. A typical profile for brain is shown in Figure 1. Although 99% of the protein, fraction H1, does not bind to the heparin column, essentially all of the mitogenic activity is retained, as measured by 3T3 IdU incorporation (Table I). Elution of the column with 1.0 M NaCl results in an elution profile

Table I: Purification of Mitogenic Activity from Bovine Brain[a]

step	protein (mg)	3T3 units
ammonium sulfate extract	5400	2×10^7 (100%)
CM-Sephadex		
CMF1	4120	1×10^5
CMF2	700	1×10^6
CMF3	320	1×10^7 (50%)
heparin affinity		
H1	300	2×10^5
H2A	1.5	7×10^5
H2B (HGFα)	0.40	7×10^6 (35%)
H3 (HGFβ)	0.02	1.6×10^6 (8%)

[a] 1 kg of starting tissue.

consisting of a sharp absorbance peak, fraction H2A, followed by a second broader peak, fraction H2B. Elution of the column with 2.0 M NaCl yields a single small absorbance peak, fraction H3. Very strong mitogenic activity for 3T3 cells is eluted with 1.0 M NaCl. Importantly, this activity correlates with the second peak of protein absorbance, fraction H2B, rather than the initial peak (Figure 1). Subsequent elution with 2.0 M NaCl results in a second peak of mitogenic activity (Figure 1). Cloned bovine capillary endothelial cells also were examined as target cells. Both mitogens stimulate IdU incorporation into capillary endothelium (Figure 1).

SDS–PAGE of fractions H2B and H3 from brain and hypothalamus is shown in Figure 2. The mitogen from either tissue eluted with 1.0 M NaCl (HGFα) is a M_r 16000 doublet, while that eluted with 2.0 M NaCl (HGFβ) is a single band of M_r 18000. The yield of HGFα and HGFβ from either tissue is 0.4 and 0.02 mg/kg of tissue, respectively (Table I). Dose–response studies on confluent 3T3 cells indicate that half-maximal stimulation of IdU incorporation by HGFα and HGFβ is achieved at concentrations of 400 and 80 pg/mL, respectively (data not shown).

To desalt both proteins into volatile buffers suitable for further chemical analysis, each was applied directly to a reversed-phase C3 HPLC column and eluted with a linear gradient of acetonitrile at a flow rate of 2%/min. The HGFα from either tissue elutes as a single sharp peak at an acetonitrile concentration of 38%. The HGFβ from either tissue elutes as a single somewhat broader peak at an acetonitrile concentration of 33%.

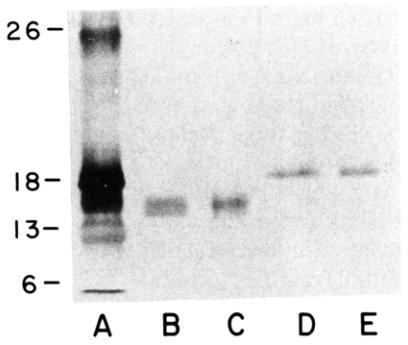

FIGURE 2: SDS–PAGE of peak H2B from brain (lane B) and hypothalamus (lane C) and peak H3 from brain (lane D) and hypothalamus (lane E). Approximately 100 ng of each sample was applied to a 12.5% polyacylamide gel with a 5% stacking gel and silver stained as described under Experimental Procedures. Molecular weight markers (Bethesda Research Laboratories, low M_r standards) are in lane A. Their molecular weights ($\times 10^{-3}$) are indicated at the left.

Bioassays and amino acid analyses were performed across the protein peaks eluted from the C3 column. For each mitogen 3T3 stimulation correlated exactly and solely with the protein peak. As shown in Table II, the amino acid compositions of the HGFα's from either tissue are identical, within experimental limits, and indistinguishable from that of acidic brain FGF (Thomas et al., 1984). Consistent with this result, elution of the C3 column with a shallow gradient of acetonitrile resolves HGFα into two peaks with almost identical amino acid compositions (data not shown), indicating that the doublet seen by SDS–PAGE represents microheterogeneous forms of the same protein, exactly as described for acidic brain FGF (Thomas et al., 1984). The amino acid compositions of the HGFβ's isolated from either tissue also are the same and distinct both from that of HGFα and from that of bovine myelin basic protein (Table II).

Discussion

Recent work has shown that heparin affinity chromatography provides a powerful means to purify endothelial mitogens (Shing et al., 1984; Klagsbrun & Shing, 1984a,b; D'Amore & Klagsbrun, 1984). A rat chondrosarcoma-derived growth factor has been purified to homogeneity by this means (Shing et al., 1984), as has a growth factor found in bovine cartilage (Sullivan & Klagsbrun, 1984). Moreover, the growth factors

Table II: Amino Acid Compositions of HGF's[a]

amino acid	acidic brain FGF[b]	HGFα[c] brain	HGFα[c] hypothalamus	HGFβ[d] brain	HGFβ[d] hypothalamus	myelin basic protein[e]
Asx	14	14.5 (15)	15.1 (15)	14.1 (14)	13.5 (14)	11
Thr	9	8.9 (9)	8.8 (9)	5.2 (5)	5.1 (5)	7
Ser	10	9.1 (9)	9.6 (10)	10.3 (10)	10.4 (10)	19
Glx	16	17.7 (18)	17.6 (18)	13.6 (14)	14.8 (15)	10
Pro	7	7.2 (7)	7.8 (8)	11.9 (12)	11.8 (12)	12
Gly	14	14.3 (14)	16.4 (16)	17.8 (18)	18.9 (19)	25
Ala	5	4.8 (5)	5.1 (5)	9.4 (9)	9.4 (9)	14
Cys	4	ND[f]	ND	ND	ND	0
Val	5	4.3 (4)	4.4 (4)	6.0 (6)	5.9 (6)	3
Met	1	1.2 (1)	1.3 (1)	2.5 (3)	2.5 (3)	2
Ile	6	6.1 (6)	5.6 (6)	3.3 (3)	3.2 (3)	3
Leu	19	19.3 (19)	20.4 (20)	13.0 (13)	13.4 (13)	10
Tyr	7	6.8 (7)	7.3 (7)	6.7 (7)	6.5 (7)	4
Phe	7	7.0 (7)	7.0 (7)	8.1 (8)	7.9 (8)	8
His	5	5.5 (6)	5.4 (5)	3.3 (3)	3.3 (3)	10
Lys	13	13.3 (13)	12.9 (13)	14.1 (14)	14.1 (14)	13
Arg	6	5.4 (5)	5.6 (6)	11.1 (11)	10.8 (11)	18
Trp	1	ND	ND	ND	ND	1
total	149	149	150	150	152	170

[a] 24-h hydrolysates of 10–20 pmol of protein. Nearest integer values are in parentheses. [b] From Thomas et al. (1984). [c] Based on a phenylalanine content of 7. [d] Based on M_r 17000. [e] From Dayhoff (1972). [f] ND, not determined.

present in bovine neural tissue are adsorbed completely to heparin–Sepharose (Klagsbrun & Shing, 1984a,b). Elution of heparin columns with salt gradients, in conjunction with isoelectric focusing studies, shows clearly that commercial extracts of bovine hypothalamus contain both an anionic and a cationic mitogen, eluting at 1.0 and 1.5 M NaCl, respectively (Klagsbrun & Shing, 1984b). Moreover, extracts of bovine brain also contain two mitogens that elute from heparin at similar salt concentrations (Klagsbrun & Shing, 1984b). In contrast, partially purified pituitary FGF, agreed to be a cationic mitogen (Lemmon & Bradshaw, 1983), elutes from heparin at 1.5 M NaCl. Finally, bovine RDGF, which isoelectric focusing studies show to be anionic (D'Amore & Klagsbrun, 1984), elutes from heparin at 1.0 M NaCl. These results suggest that the growth factors in neural tissue can be grouped into two classes: mitogens that elute from heparin at about 1 M sodium chloride (HGFα's), likely all anionic, and mitogens that elute at about 1.5 M sodium chloride (HGFβ's), likely all cationic. However, the structural relationship between the mitogens present in these tissues remained undefined.

To extend these results, large-scale extractions of bovine brain and hypothalamus were performed according to the method of Gospodarowicz et al. (1982), coupled with heparin affinity chromatography. Two growth factors were purified to homogeneity from either bovine hypothalamus or brain. The two mitogens, one eluted from heparin with 1.0 M NaCl (HGFα) and the other with 2.0 M NaCl (HGFβ), are obtained pure in only three steps with yields of 0.4 and 0.02 mg/kg of tissue, respectively. Both stimulate the growth of 3T3 cells and bovine capillary endothelial cells.

The HGFα purified from either tissue is the same, on the basis of the elution behavior on heparin affinity and reversed-phase C3 columns, amino acid composition, and SDS–PAGE. Moreover, its amino acid composition and molecular weight show that it is identical with the acidic fibroblast growth factor recently isolated from bovine brain by a multistep chromatographic procedure (Thomas et al., 1984). These results confirm that HGFα is anionic.

Acidic endothelial cell growth factors have been partially purified from both bovine hypothalamus (ECGF; Maciag et al., 1979, 1982) and bovine retina (RDGF; D'Amore et al., 1981). However, their relationship to the mitogens found in other neural tissues was unclear. The acidic mitogen present in commercial extracts of hypothalamus binds to heparin–Sepharose and elutes at about 1 M NaCl, as does RDGF (Klagsbrun & Shing, 1984a,b; D'Amore & Klagsbrun, 1984). Moreover, both growth factors behave similarly upon isoelectric focusing and gel filtration and display similar biological activities, strongly suggesting that the two mitogens are identical (D'Amore & Klagsbrun, 1984). The results given here establish that hypothalamic ECGF and acidic brain FGF are in fact the same and imply that there is only one acidic mitogen in bovine neural tissue.

The HGFβ's isolated from either brain or hypothalamus are also identical (Figure 2, Table II). The apparent molecular weight is 18 000 by SDS–PAGE and the amino acid composition is distinct from HGFα. Since isoelectric focusing studies show that the mitogen in hypothalamus that elutes from heparin–Sepharose at 1.5 M NaCl is cationic (Klagsbrun & Shing, 1984b), these results indicate that bovine neural HGFβ is a cationic mitogen.

These results address the issue of the identity of bovine brain fibroblast growth factor. Brain FGF was suggested to be a cationic protein derived from proteolytic fragments of myelin basic protein (Westall et al., 1978). Subsequently, it was suggested not only that the mitogen was unrelated to myelin basic protein but also that it was acidic (Thomas et al., 1980). Recently, an acidic form of FGF has been purified to homogeneity by conventional multistep chromatographic procedures (Thomas et al., 1984), confirming previous observations (Thomas et al., 1980; Lemmon et al., 1982) that at least part of the growth factor activity in bovine brain is due to an anionic mitogen. Nevertheless, a cationic mitogen also has been observed consistently in neural tissue (Lemmon et al., 1982; Gospodarowicz et al., 1982; Lemmon & Bradshaw, 1983; Klagsbrun & Shing, 1984b). The results presented here confirm the existence in both brain and hypothalamus of a cationic growth factor with an amino acid composition clearly distinct from that of myelin basic protein (Table II).

These and other results (Klagsbrun & Shing, 1984b) show that past difficulties in defining the nature of the mitogens in bovine neural tissue can be attributed to multiple factors. Most importantly, while both anionic and cationic mitogens are indeed present in neural tissue, the efficiency with which they can be extracted during tissue homogenization varies considerably. Extraction of bovine hypothalamus at neutral pH and low ionic strength largely extracts the anionic mitogen (Maciag et al., 1979, 1982). However, the quantity of cationic mitogen obtained from hypothalamus after salt extraction at neutral pH increases substantially with increasing salt concentration.[2] Moreover, digestion of hypothalamic tissue at neutral pH and low ionic strength with bacterial collagenase also increases the yield of cationic mitogen.[2] Such results suggest that this mitogen is complexed to the extracellular matrix. As suggested previously (Lemmon et al., 1982), small but critical changes in pH, volumes, salt concentration, effectiveness of homogenization, etc. markedly change the ratio of the two mitogens. In addition, a variety of target cells have been used. The isolation of acidic brain FGF (HGFα) has relied largely on stimulation of murine 3T3 cells, a fibroblastic cell line (Thomas et al., 1980, 1984; Lemmon et al., 1982). In contrast, cationic brain FGF (HGFβ) has been isolated on the basis of the proliferation of bovine aortic endothelial cells (Gosporarowicz et al., 1978, 1982). Additionally, hypothalamic ECGF (HGFα) has been isolated on the basis of the growth of human umbilical vein endothelium (Maciag et al., 1979, 1982). A wide variation in sensitivity of different endothelial cell clones to FGF has been clearly demonstrated (Longenecker et al., 1983) and may explain some of the observed differences in target cell specificity. As shown here and elsewhere (Klagsbrun & Shing, 1984b), both murine fibroblast 3T3 and bovine capillary endothelial cells respond to both mitogens.

In summary, the mitogenic activity present in bovine neural tissue is likely due to only two growth factors, one anionic (acidic brain FGF, ECGF, RDGF) and one cationic (pituitary

here been renamed heparin-binding growth factors, HGFα and HGFβ, respectively, because of their broad target cell specificity and their ability to bind to heparin, an interaction which is likely of considerable significance in vivo. The ability to isolate large quantities of these growth factors from bovine neural tissue should allow the rapid resolution of a number of critical issues. These include the structural relationship between the two classes of factors and between bovine neural HGFβ and the cationic mitogens of similar molecular weight isolated from bovine cartilage (Sullivan & Klagsbrun, 1984) and a rat chondrosarcoma (Shing et al., 1984), the nature of

[2] M. Klagsbrun, personal communication.

the heparin/protein interaction, the structure of the heparin fragments responsible for binding, and the nature of the cell receptor. Application of the methods presented here to placental tissue has already allowed the purification to homogeneity of a human HGFβ.[3] Initial results on the structural and functional characterization of this family of growth factors from both human and bovine sources will be reported shortly.

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[3] R. R. Lobb, unpublished experiments.

Interstrand Psoralen Cross-Links Do Not Introduce Appreciable Bends in DNA†

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ABSTRACT: Analysis of the X-ray crystallographic structure of an 8-methoxypsoralen–thymine monoadduct has led to the suggestion that psoralen cross-links would bend DNA by as much as 70° [Peckler, S., Graves, B., Kanne, D., Rapoport, H., Hearst, J. E., & Kim, S.-H. (1982) *J. Mol. Biol. 162*, 157–172]. DNA can exist in a stably bent configuration in solution as recently demonstrated from analysis of polyacrylamide gel electrophoresis and differential decay of birefringence. Using these techniques, we have investigated the structure of DNA cross-linked with 8-methoxypsoralen and 4,5′,8-trimethylpsoralen. The results are not consistent with cross-links introducing any appreciable stable bend in double-stranded DNA molecules. Results suggest that photobound 4,5′,8-trimethylpsoralen molecules lengthen DNA by the equivalent of about one base pair per photobound adduct. We have also determined that 4,5′,8-trimethylpsoralen cross-links are introduced preferentially into 5′-TA compared to 5′-AT DNA sequences.

Recent evidence has demonstrated convincingly that DNA can exist in solution in a stably bent conformation (Wu & Crothers, 1984; Hagerman, 1984a). DNA can bend as a result of the inherent conformation of particular sequence arrangements of DNA as in trypanosome kinetoplast DNA (Wu & Crothers, 1984; Hagerman, 1984a). In addition, DNA may be bent by association with proteins as demonstrated for the CAP–*lac* operator DNA binding interaction (Wu & Crothers, 1984). Bent DNA adds another dimension of complexity, in addition to cruciforms and regions of left-handed Z-DNA, to the structure of DNA. It is possible that these alternate helical conformations of DNA, compared to a linear B-form DNA,

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