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# Structural and Functional Characterization of Full-Length Heparin-Binding Growth Associated Molecule

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Heparin-binding growth-associated molecule (HB-GAM) was purified from adult bovine brain and chicken heart. The yield of HB-GAM is increased by 5- to 10-fold when 250 mM NaCl is added to the homogenization buffer, indicating that HB-GAM may exist as a complex with an insoluble component of the tissue. The complete amino acid sequence of the brain-derived HB-GAM was established by automated Edman degradation of the intact protein and chemically or enzymatically derived fragments. The mass of bovine HB-GAM as determined by plasma desorption time-of-flight mass spectrometry is 15 291 mass units, which compares favorably with the calculated mass of 15 289 based on the amino acid sequence. Therefore, HB-GAM has not undergone any major post-translational modifications other than cleavage of the signal peptide. These results indicate that previous amino acid sequence analysis of this protein was carried out using truncated HB-GAM. Full-length HB-GAM is not a mitogen for Balb/3T3 clone A31, Balb MK, NRK, or human umbilical vein endothelial cells. HB-GAM does, however, have adhesive properties and neurite extension activity for chick embryo cerebral cortical derived neurons when presented to these cells as a substrate. HB-GAM had little neurite extension activity when presented as a soluble factor.

## INTRODUCTION

Heparin-binding growth-associated molecule (HB-GAM)[1] (also referred to as ECGF-LP, p18, HBGF-8, HBNF, or pleotrophin) is a basic cysteine-rich heparin-binding protein that has been isolated from brain (Burgess *et al.*, 1985; Rauvala, 1989; Kuo *et al.*, 1990) and placenta (Milner *et al.*, 1989). The cDNA that encodes this protein has been cloned and sequenced from several species (Li *et al.*, 1990; Merenmies and Rauvala, 1990). This protein is expressed in a temporal- and spacial-specific manner in the developing embryo and is expressed intensely in the brain of postnatal rats (Rauvala, 1989). HB-GAM is related structurally to MK, a protein expressed by embryonal carcinoma cells after treatment with retinoic acid (Kadomatsu *et al.*, 1988) and to a protein isolated from chick embryo basement membranes (Vigny *et al.*, 1989; Urios *et al.*, 1991). Several features are common among these proteins. For example, they are all basic proteins rich in lysine residues and they have a high apparent affinity for heparin. The relative positions of 10 cysteine residues are conserved and based on the cDNA sequences; the open reading frames encode consensus signal peptide sequences that would allow these proteins to be secreted.

During the course of purification of bovine brain-derived acidic fibroblast growth factor (FGF-1),[2] originally referred to as endothelial cell growth factor, we identified a heparin-binding protein that was related immunologically and had an apparent molecular weight similar to FGF-1; hence, it was named endothelial cell growth factor-like protein (ECGF-LP) (Burgess *et al.*,

[1] Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ECGF-LP, endothelial cell growth factor-like protein; FGF-1, acidic fibroblast growth factor; HB-GAM, heparin-binding growth-associated molecule; HPLC, high-pressure liquid chromatography; NTF, neurotrophic factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RI-HB, retinoic acid-induced heparin binding; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

[2] The abbreviations used for fibroblast growth factors are based on the conclusions of the New York Academy of Science conference, La Jolla, California, 1991.

1985). Further analysis revealed that this protein had a high content of lysine residues, and amino-terminal sequence analysis indicated that this protein was not related structurally to members of the FGF family. Recently, the cDNA sequence of rat HB-GAM was reported (Merenmies and Rauvala, 1990) and the deduced amino acid sequence from this cDNA revealed that ECGF-LP was the bovine homolog of HB-GAM.

The cDNA sequence of HB-GAM contains an open reading frame that encodes for a putative signal peptide that would be cleaved during processing leaving a 136 amino acid polypeptide that may be secreted. To date, amino acid sequence analysis has failed to identify a 136 amino acid form of the protein (Kuo *et al.*, 1990; Bohlen *et al.*, 1991). Rather, the complete amino acid sequence of bovine HB-GAM was reported to consist of 119 amino acids (Kuo *et al.*, 1990), suggesting the possibility that proteolysis of the carboxyl-terminus had occurred. More recently, the documented amino acid sequence of a 114 amino acid form of HB-GAM was reported (Bohlen *et al.*, 1991). In that report, the amino acid composition analysis indicated that a 136 residue form of the protein may exist; however, amino acid sequence extending beyond residue 114 was not obtained.

The two biological activities reported for HB-GAM are a mitogen for mouse or rat fibroblasts and a neurite extension factor for rat brain neurons and a rat pheochromocytoma cell line (PC-12). However, there has not been a consensus in the literature with regard to the assignment of these activities. Ravaula (1989) determined that rat brain-derived HB-GAM was a neurite extension factor for rat neurons in vitro. Milner *et al.* (1989) described the isolation of HB-GAM from an acetone powder preparation of bovine placenta and reported it to be an acid labile mitogen for NIH 3T3 cells with a potency equivalent to that of FGF-1. However, sufficient evidence was not presented to exclude the possibility of contamination by other heparin-binding growth factors such as basic fibroblast growth factor. Indeed, ~50% of the mitogenic activity in their preparation did not correlate with the elution position of the major UV absorbing peak (from the final purification step) but could be found in the side fractions of this peak (Milner *et al.*, 1989). Subsequent experiments by Li *et al.* (1990) showed that when the cloned gene is transiently expressed in COS cells, the cell extract has relatively little mitogenic activity on the basis of the level of expression of recombinant HB-GAM. Other studies that reported mitogenic activity of bovine HB-GAM (Bohlen *et al.*, 1988; Bohlen and Gautschi, 1989) have recently been retracted (Bohlen *et al.*, 1991). We reported previously (Burgess *et al.*, 1985) that a protein now known to be identical to bovine brain-derived HB-GAM was not a mitogen for mouse lung capillary endothelial cells. Similarly, it was reported by Kuo *et al.* (1990) that their preparation of HB-GAM was not a mitogen for NIH 3T3 cells but is a neurite extension factor for PC-12 cells. However, it is possible that the lack of mitogenic activity in these preparations is related to carboxyl-terminal truncations (Kuo *et al.*, 1990; Bohlen *et al.*, 1991) or to acidic conditions used during purification (Burgess *et al.*, 1985). To resolve these questions we extended our studies on the isolation and characterization of this protein.

In this report we describe the complete primary structure of bovine and avian HB-GAM. On the basis of the primary structure, we show that these mature proteins consist of 136 or 135 amino acids, respectively, and present evidence that the truncated forms of HB-GAM isolated previously (Kuo *et al.*, 1990; Bohlen *et al.*, 1991) are an artifact of purification rather than post-translational modification. We also demonstrate its neurotrophic activity on chick embryo cerebral cortical neurons and its lack of mitogenic activity on BALB/3T3, BALB/MK, NRK, and human umbilical vein endothelial cells as compared with FGF-1. We show that HB-GAM is present in adult tissues of non-neuronal origin and present direct evidence that HB-GAM has not undergone any significant post-translational modification other than cleavage of the amino-terminal signal peptide. In contrast to previous reports (Burgess *et al.*, 1985; Kuo *et al.*, 1990; Bohlen *et al.*, 1991), the lack of mitogenic activity of our preparations of HB-GAM cannot be attributed to acidic treatment or carboxyl-terminal truncations of the protein.

## MATERIALS AND METHODS

### *Purification*

HB-GAM was purified essentially as described (Burgess *et al.*, 1985) with some modifications. Six adult bovine brains were homogenized in 1.3 volumes (wt/vol) of 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 50 mM EDTA, 250 mM NaCl. The homogenate was clarified of insoluble material by centrifugation at 13 500 × *g* for 60 min. Ammonium sulfate was added to the supernatant to 50% saturation, stirred for 60 min, centrifuged at 13 500 × *g* for 60 min, and the pellet was discarded. Additional ammonium sulfate was added to 95% saturation, stirred for 60 min, and centrifuged at 13 500 × *g* for 60 min. The resulting pellet was resuspended in 10 volumes of H/S buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA) and stirred with 40 ml of heparin-Sepharose (Pharmacia, Piscataway, NJ) equilibrated with H/S buffer. After 60 min the heparin-Sepharose was washed on a scintered glass funnel with 1000 ml of H/S buffer and then with 1000 ml of H/S buffer containing 500 mM NaCl. The resin was transferred to a 1.5-cm diameter column and bound proteins were eluted with a gradient from 0.5 to 1.5 M NaCl and 10-ml fractions were collected.

The fractions that eluted between 0.8 and 1.1 M NaCl were pooled, diluted 10-fold in 50 mM sodium phosphate buffer, pH 6.0, and fractionated further by ion exchange chromatography using a Waters 840 HPLC system (Waters, Medford, MA) and a 5 mm × 5 cm MONO-S column (Pharmacia) equilibrated in the same buffer. Proteins were eluted with a linear gradient from 0 to 1.0 M NaCl over 50 min. Final purification of HB-GAM was achieved by ion exchange chromatography on an Aquapore (Browlee Labs, Santa Clara, CA) CX-300 2.1 mm × 10 cm column equilibrated with 50 mM sodium phosphate pH 7.0 and eluted as described above. Purification of HB-GAM from chicken heart was done as previously described for the purification of FGF-1 from bovine heart (Sasaki *et al.*, 1989). Recombinant human

FGF-1 was used in the bioassays and was purified as described previously (Burgess *et al.*, 1990). Briefly, cultures of *Escherichia coli* bearing a plasmid that encodes residues 1–154 of human FGF-1 (kindly provided by Michael Jaye: Rhone-Poulenc Rorer, King of Prussia, PA) were grown and FGF-1 was purified by heparin-Sepharose affinity chromatography and reversed-phase chromatography. These preparations were judged to be >95% pure on the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### *Determination of the Primary Structure*

Before enzymatic digestion, the cysteine residues of HB-GAM were modified. Briefly, reduction was carried out with 59 mM 2-mercaptoethanol in 6 M guanidine-HCl, 250 mM Tris-HCl, pH 8.5, 1 mM EDTA at 37 μC for 60 min. 4-Vinyl-pyridine was then added to a final concentration of 440 mM and incubated at room temperature for 90 min. Enzymatic digestions of 1–3 nmol of HB-GAM with endoproteinase Lys-C, endoproteinase Glu-C, or endoproteinase Asp-N were carried out according to the manufacturers instructions at a ratio of 1:10 enzyme to protein (wt/wt). The buffer used in the digestions with endoproteinase Glu-C was 25 mM ammonium bicarbonate (pH 7.8) to limit this enzyme's specificity to cleavage primarily at the carboxyl-terminal side of glutamic acid. Additionally, all digestion buffers contained 10% (vol/vol) acetonitrile. HB-GAM was also cleaved at methionine residues by cyanogen bromide. Peptides generated by enzymatic or chemical cleavage were purified on an Aquapore 1 mm × 10 cm OD-300 column with an Applied Biosystems (Foster City, CA) model 130A high-pressure liquid chromatography (HPLC) system.

The amino acid sequence of the peptides was determined by automated Edman degradation on an Applied Biosystems model 477A protein sequencer with a model 120A microbore HPLC system for on-line analysis of phenylthiohydantoin amino acids. An Applied Biosystems model 473A protein sequencer was also used for sequencing peptide fragments and data from this instrument was analyzed using the model 610A data analysis software version 1.2 (Applied Biosystems).

The mass of intact HB-GAM was determined by plasma desorption mass spectrometry with a Bio Ion 20 (Bio Ion Division, Applied Biosystems Inc., Uppsala, Sweden) time-of-flight mass spectrometer (Sundqvist *et al.*, 1984). A 200-pmol aliquot of HB-GAM was applied to aluminized Mylar (Bioion Division Applied Biosystems, Uppsala, Sweden) that was electrosprayed previously with 100 μg of nitrocellulose (Jonsson *et al.*, 1986). The sample disc was spin-dried and washed with 20 μl of 0.1% trifluoroacetic acid (Roepstorff *et al.*, 1987). Ionization of the protein was achieved by exposure to $^{252}Cf$ fission fragments (Sundqvist *et al.*, 1984). Spectra were collected for 20–24 h at 20 kV accelerating potential. Spectra were calibrated with hydrogen and sodium ions.

The number of free sulfhydryl groups was determined by incubating 200-pmol aliquots of native HB-GAM in 6 M guanidine-HCl 0.25 M Tris-HCl, pH 8.5, 1 mM EDTA in the presence of 440 mM 4-vinylpyridine for 90 min at room temperature. As a control experiment, HB-GAM was first reduced by incubation in the above buffer containing 50 mM 2-mercaptoethanol for 60 min at 37 °C and subsequently incubated with 4-vinyl-pyridine as above. The reaction mixtures were desalted on a 1 mm × 10 cm Aquapore OD-300 column and dried with a Speed Vac (Savant Instruments, Farmingdale, NY) concentrator. The amino acid compositions of the reduced/pyridylethylated and unreduced/pyridylethylated samples were determined after hydrolysis in 6 M HCl at 150° for 60 min. The hydrolyzed samples were derivitized with phenylisothiocyanate and the resulting PTC-amino acids were analyzed on a model 840 PICOTAG amino acid analysis system (Waters, Medford, MA).

### *Bioassays*

The ability of HB-GAM to stimulate DNA synthesis was determined using Balb/3T3 clone A31, NRK 49F (American Type Culture Collection, Rockville, MD), or BALB/MK (Weissman and Aaronson, 1983) cells. Either Balb/3T3 or NRK cells were grown to 50% confluence in 48-well cluster dishes containing Dulbecco's modified Eagle's medium (DMEM):F12 (1:1 mixture, Irvine Scientific, Santa Anna, CA) and 10% bovine calf serum (Inovar, Gaithersburg, MD) at 37°C, 5% $CO_2$ in a humidified atmosphere. The growth media was removed and replaced with starvation medium (DMEM:F12 1:1 mixture, 0.5% bovine calf serum, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.2). The cells were incubated as above in this medium for an additional 72 h. Dilutions of HB-GAM or FGF-1 were added to the wells and incubated at 37°C for 16 h. The cells were labeled with 0.5 μCi/ml of $^3H$-methyl-thymidine (25 mCi/mmol, Amersham, Arlington Heights, IL) for an additional 4 h. Assays with BALB/MK cells were performed as previously described (Rubin *et al.*, 1989). The media was aspirated, and $^3H$-methyl-thymidine that had incorporated into DNA was precipitated by adding 100 μl of ice-cold 10% trichloroacetic acid to each well and incubating on ice for 15 min. DNA was solubilized with 1.0 M NaOH at 37°C for 15 min. The amount of radioactivity in each sample was determined by scintillation counting. All assays were performed in triplicate.

The human umbilical vein endothelial cells used in the growth assays were provided by T. Maciag (American Red Cross, Rockville, MD). The cells were maintained on fibronectin-coated tissue culture dishes in medium 199 (Irvine Scientific, Santa Ana, CA) containing 20% (vol/vol) fetal bovine serum, one times antibiotic-antimycotic (GIBCO, Grand Island, NY), 5 units/ml heparin (Upjohn, Kalamazoo, MI), 10 ng/ml human recombinant FGF-1, 5 μg/ml insulin, 4.8 ng/ml selenium, and 5 μg/ml transferrin. For the growth assays, the cells were trypsinized and seeded at $5.6 \times 10^4$ cells/well in 12-well cluster dishes (Corning, Silver Spring, MD) that were coated with fibronectin (5 μg/cm$^2$) in the above media without FGF-1. The indicated amounts of HB-GAM or FGF-1 were added to wells in triplicate with or without 5 units/ml heparin. The medium and growth factors were changed every 2 d, and after 6 d the cells were trypsinized and counted with a hemacytometer.

The neurotrophic activity of HB-GAM was tested on dissociated chicken embryo cerebral cortical neurons (Kligman, 1982). Briefly, the cerebral cortex from four day-7 (stage 31–32) chicken embryos was dissected and treated with calcium-magnesium-free phosphate-buffered saline (PBS) and the cells were suspended by gentle trituration with a pasteur pipet and counted. Neurons were plated at a density of $1 \times 10^4$ cells/cm$^2$ on 35-mm tissue culture plates that had been untreated or coated previously with 0.25 ml of either 25 μg/ml of poly-L-lysine (Sigma, St. Louis, MO) or 8 μg/ml of HB-GAM dissolved in sterile water. The plates were rinsed with sterile distilled water three times before adding the cells. After 42 h incubation in serum free medium (Ham's F12 base, 5 μg/ml insulin, 5 μg/ml transferrin, 20 nM progesterone, [Sigma] 100 μM putrescine, 30 nM sodium selenite [Aldrich, Milwaukee, WI]) at 37°C the cells were fixed in 1% glutaraldehyde in PBS and counted on a Nikon (Garden City, NY) Diaphot inverted microscope with a 20× objective under phase contrast. Neurite outgrowth was assessed by counting 100–200 cells in four fields of each culture plate. All assays were performed in duplicate.

## RESULTS

The purification methods used here resulted in the recovery of relatively large quantities of intact HB-GAM from bovine brain and chicken heart and indicated its presence in adult tissues of neural and non-neural origin. The yield of HB-GAM from bovine brain was significantly higher when 250 mM NaCl was added to the homogenization buffer; the yield was 120 ng/g (wet wt) and 17 ng/g (wet wt) with or without added NaCl, respectively. HB-GAM was also isolated from chicken heart by a chloroform/methanol procedure (Sasaki *et al.*, 1989). The yield from this source was 360 ng/g (wet

wt). These yields are significantly higher relative to the yield of other heparin-binding growth factors isolated from these tissues but is lower than the amount of HB-GAM isolated from postnatal rat brain (Rauvala, 1989).

The apparent molecular weight of HB-GAM as estimated by SDS-PAGE is ~18 000 Da (Figure 1), and the amino acid composition indicated it has a high content of lysine and cysteine residues. The number of free sulfhydryl groups was determined after reaction of HB-GAM with 4-vinyl-pyridine. Analysis for the content of pyridylethylated cysteine residues after acid hydrolysis and derivitization with phenylisothiocyanate revealed 4 pyridylethylcysteine residues present in unreduced HB-GAM and 10 pyridylethylcysteine residues in reduced HB-GAM. Because HB-GAM contains 10 cysteine residues, these results indicate that 6 of the 10 cysteine residues have formed disulfide bonds.

In certain cases the generation of a truncated form of HB-GAM was observed during the course of its purification. The quantitative conversion of the 18 000-Da form to a 15 000-Da form (Figure 1) was observed if HB-GAM was batch eluted from heparin-Sepharose with 1.5 M NaCl, diluted into 50 mM phosphate buffer, pH 6.0, and subsequently purified by ion-exchange chromatography. This conversion did not occur if HB-GAM was either eluted from heparin-Sepharose with a NaCl gradient or if it was purified immediately by reversed-phase HPLC.

The complete amino acid sequence of bovine HB-GAM was established by a combination of amino terminal sequencing and sequencing of peptides generated by either enzymatic or chemical cleavage. From the sequences of overlapping peptide fragments it was determined that bovine HB-GAM consisted of 136 amino acids (Figure 2). The calculated mass of this protein was 15 289 Da, which is considerably less than the apparent molecular weight estimated from SDS-PAGE (Figure 1). To determine whether there were any major post-translational modifications to the protein and to verify that the derived sequence was indeed complete, the mass of HB-GAM was determined by plasma desorption time-of-flight mass spectrometry. The mass was determined to be 15 291 mass units on the basis of the $(M + 3H)^{3+}$ ion. Therefore, we conclude that HB-GAM has not undergone any major post-translational modifications that would have added significant mass to the protein and that the amino acid sequence shown in Figure 2 is complete.

The amino acid sequence of chicken HB-GAM was also determined as described above; comparison of the bovine and chicken sequences revealed seven amino acid substitutions and one amino acid deletion (Figure 2). Serine$^{121}$ was not present in the chicken HB-GAM; thus, this polypeptide consists of 135 amino acids. The substitution of serine$^{115}$ in the bovine sequence with a proline in the chicken sequence is consistent with the residue present in that position of the rat and human

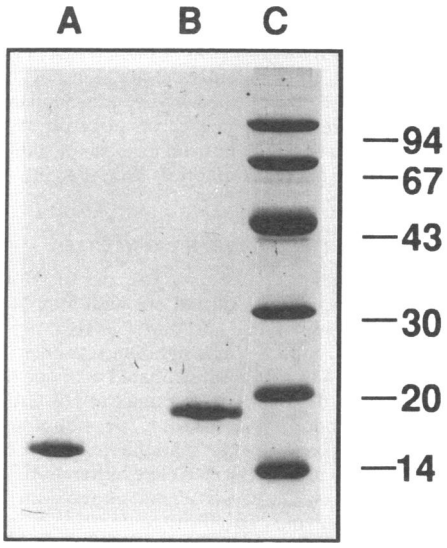


**Figure 1.** SDS-PAGE of intact and truncated HB-GAM. Samples were subjected to electrophoresis in a 13% acrylamide, 0.4% *N,N'*-methylenebisacrylamide gel polymerized in a Hoefer minigel apparatus with the buffer system of Laemmli (1970). Electrophoresis was carried out at 200 V until the marker dye reached the end of the gel. Lane A, truncated HB-GAM isolated by batch elution from heparin-Sepharose and ion-exchange chromatography; lane B, intact HB-GAM isolated by gradient elution from heparin-Sepharose and ion-exchange chromatography; lane C, Pharmacia low-molecular-weight standards. The numbers to the right indicate the approximate molecular weight $\times 10^{-3}$.

sequences of HB-GAM (Li *et al.*, 1990; Merenmies and Rauvala, 1990). It was not possible to precisely identify the residue at position 119 because the signal carry over from the previous cycle (which is a glutamine) interfered with determining whether the identity of residue 119 was truly a glutamic acid residue or a glutamine residue that had undergone substantial deamidation. The identity of residue 135 (the carboxyl-terminal residue of the chicken sequence) was determined by amino acid composition of the Glu-C-derived fragment (residues 128–135). The identity of the amino acid residue at position 135 could be either an asparagine or an aspartic acid because asparagine undergoes deamidation during acid hydrolysis.

As illustrated in Figure 3A, bovine HB-GAM in the presence or absence of heparin was not mitogenic for Balb/3T3 clone A31 cells. In contrast, FGF-1 in the presence of heparin elicits a potent mitogenic response with a half maximal dose of 0.3–0.5 ng/ml. Without heparin in the assay medium, FGF-1 is significantly less potent. Figure 3, B and C show the mitogenic response of Balb/MK (a mouse epidermal keratinocyte) and NRK (normal rat kidney fibroblasts), respectively, to FGF-1 and HB-GAM. The results of these assays further demonstrate the lack of mitogenic activity of HB-GAM. HB-GAM was also tested for its ability to promote the growth of human umbilical vein endothelial cells. As

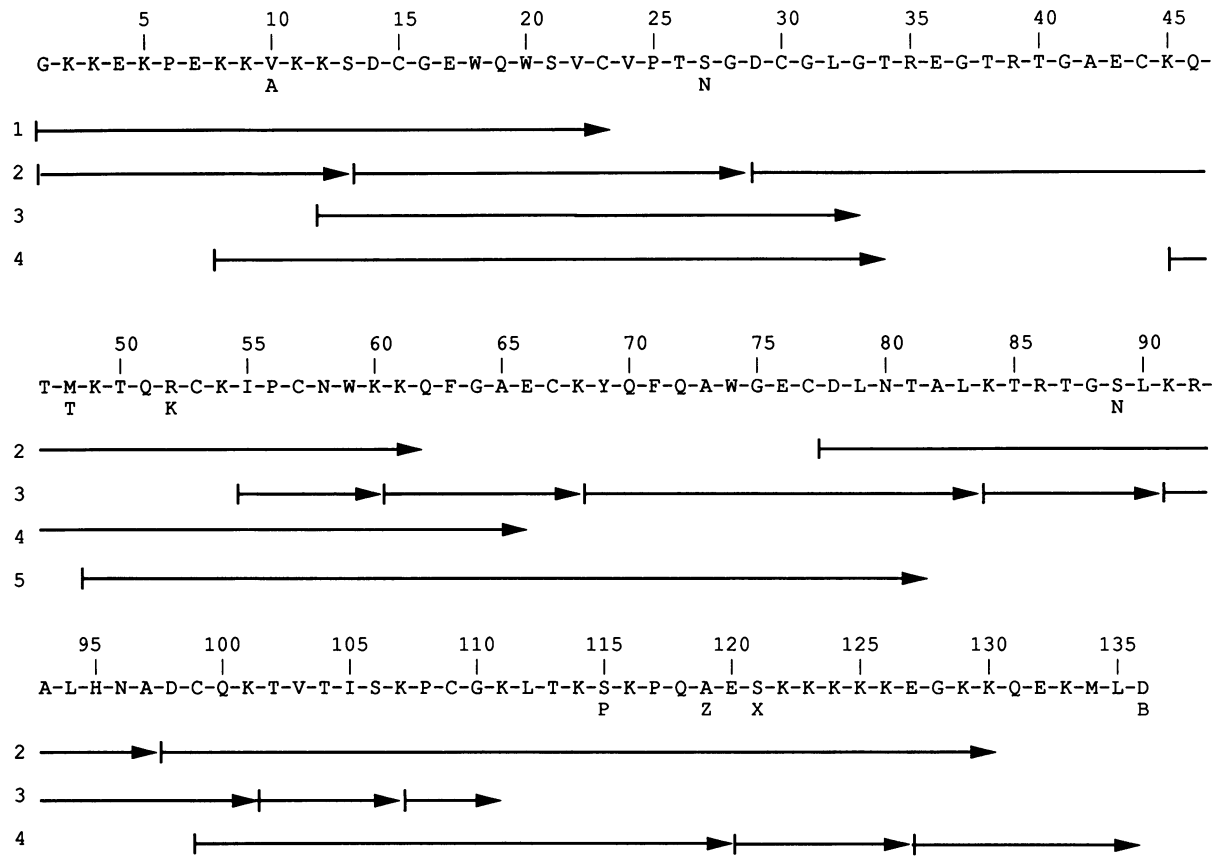


**Figure 2.** The amino acid sequence of bovine and chicken HB-GAM. The amino-terminal sequence of bovine HB-GAM and the sequences derived from cyanogen bromide and proteolytically derived fragments were aligned to give the complete 136 amino acid sequence. The amino acid sequence of chicken HB-GAM is listed under the bovine sequence with only the amino acid differences indicated. An X at position 121 indicates the residue that is deleted in the chicken sequence. The vertical lines indicate the beginning of the sequence of a fragment and the arrows indicate where sequencing of a fragment ended. The numbers to the left indicate the reagent used to generate the peptide fragments. 1, the amino-terminal sequence of intact HB-GAM; 2, endoproteinase Asp-N; 3, endoproteinase Lys-C; 4, endoproteinase Glu-C; 5, cyanogen bromide.

shown in Table 1, FGF-1 in the presence of heparin stimulated a 20-fold increase in cell number during the 6-d assay period. However, HB-GAM was not able to induce a growth response in the presence or absence of heparin. Therefore, HB-GAM (unlike FGF-1) is not a mitogen for fibroblasts, keratinocytes, or endothelial cells in vitro.

The ability of HB-GAM to act as a neurotrophic factor was tested using a chicken embryo cerebral cortical neurite outgrowth assay. When 500 ng/ml of HB-GAM was present in the assay medium and the cells were plated on untreated culture dishes, 3.6% of the neurons extended neurites (Figures 4A and 5A). There was a pronounced increase in neurite extension when the culture dish was coated with HB-GAM (Figures 4B and 5B); however, there was no significant increase in this response if in addition to coating the dish, HB-GAM was also added to the assay medium (Figures 4C and 5C). When poly-L-lysine was used as an attachment substrate and HB-GAM was added as a soluble factor, there was a small increase in neurite outgrowth compared with the untreated dish (Figures 4E and 5E). Figures 4D and 5D show the response to the negative control (poly-L-lysine coating with no soluble factor added) and Figures 4F and 5F show the response to the positive control (poly-L-lysine coating with 10 $\mu$l fetal bovine serum added as a soluble factor).

## DISCUSSION

In this report we describe the isolation and complete primary structure of HB-GAM, a highly basic heparin-binding protein from adult bovine brains or chicken hearts. Bovine HB-GAM consists of 136 amino acids; of these there are 10 cysteines and 28 lysines. Seven of the 10 cysteines are flanked by charged residues. The number of free cysteine residues in bovine HB-GAM was determined to be four, in contrast to the results reported previously (Kuo *et al.*, 1990). In that report $^{14}$C-iodoacetamide was incubated with HB-GAM for 10

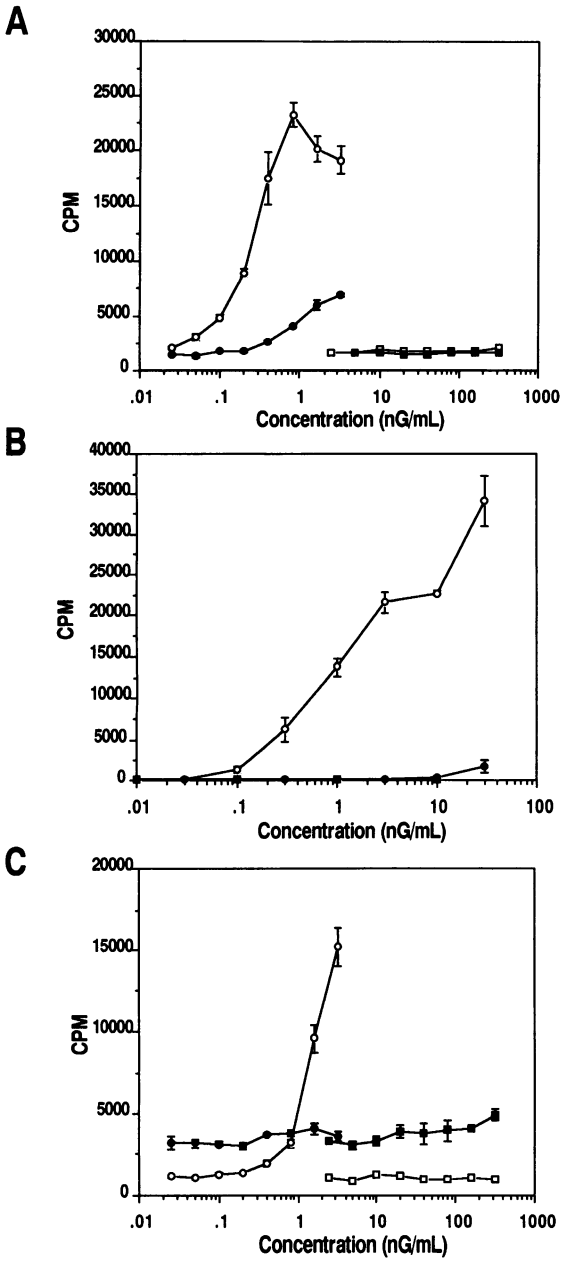


**Figure 3.** Mitogenic assay. Stimulation of DNA synthesis by HB-GAM or FGF-1. The magnitude of the response was measured by the amount of [$^3$H]thymidine that was incorporated into DNA in response to HB-GAM with 10 units/ml heparin □——□, or without heparin ■——■, and FGF-1 with heparin ○——○, or without heparin ●——●. (A) BALB/3T3 cells; (B) BALB/MK cells; (C) NRK cells.

min and no incorporation of radioactivity into the protein was observed. On the basis of those results, it was concluded that there are no free sulfhydryl groups in HB-GAM; however, the number of cysteine residues accessible to iodoacetamide after reduction was not determined. There are three potential nuclear targeting sequences based on the consensus sequence, K-R/K-X-R/K, as described previously (Chelsky *et al.*, 1989) and there are no consensus *N*-linked glycosylation sites. The mass of HB-GAM, determined by plasma desorption time-of-flight mass spectrometry, is in close agreement with the mass calculated from the amino acid sequence. Therefore, we conclude that there are no post-translational modifications to HB-GAM that would result in a significant increase in mass, and as suggested previously (Kuo *et al.*, 1990), the aberrant mobility of HB-GAM on SDS-PAGE is likely due to its inherent charge.

Brain is a rich source of heparin-binding growth factors and proteases. Any number of such proteins may copurify with HB-GAM resulting in preparations contaminated with mitogenic or proteolytic activities. Fur-

**Table 1.** Human umbilical vein endothelial cell growth assay.[a]

| | Growth factor concentration (ng/ml) | | | |
|---|---|---|---|---|
| | 0 | 1 | 10 | 100 |
| HB-GAM | 22 ± 22 | 21 ± 4 | 20 ± 2 | 16 ± 4 |
| HB-GAM + 10 U/ml heparin | ND | 21 ± 8 | 17 ± 5 | 14 ± 4 |
| FGF-1 + 10 U/ml heparin | ND | ND | 448 ± 33 | ND |

[a] Cell number/well × $10^{-3}$. Values are means ± SD. ND, not determined

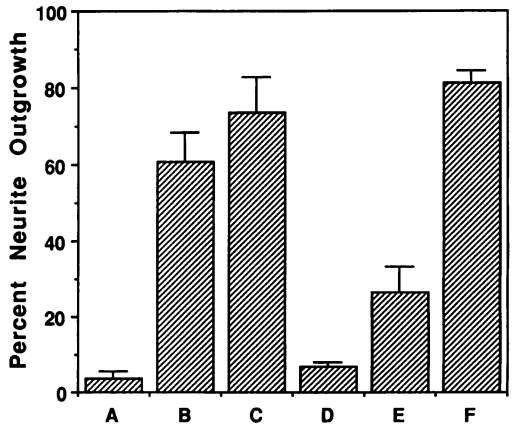


**Figure 4.** Neurite extension assay. Chicken cerebral cortex neurons were prepared from 7-d embryos (see MATERIALS AND METHODS) and plated on either HB-GAM coated, poly-L-lysine coated, or uncoated tissue culture dishes with or without HB-GAM or serum added to the culture medium. The cells were incubated at 37 μC for 42 h, fixed with 1% glutaraldehyde, and duplicate dishes scored. A positive response is a cell with at least one neurite that is twice the length of the diameter of the cell body. A, uncoated dish, 100 ng/ml HB-GAM in the medium; B, HB-GAM-coated dish, no addition to medium; C, same as B with 100 ng/ml HB-GAM in the medium; D, poly-lysine-coated dish, no addition to the medium; E, same as D with 100 ng/ml HB-GAM in the medium; F, same as D with 10% fetal calf serum in the medium.

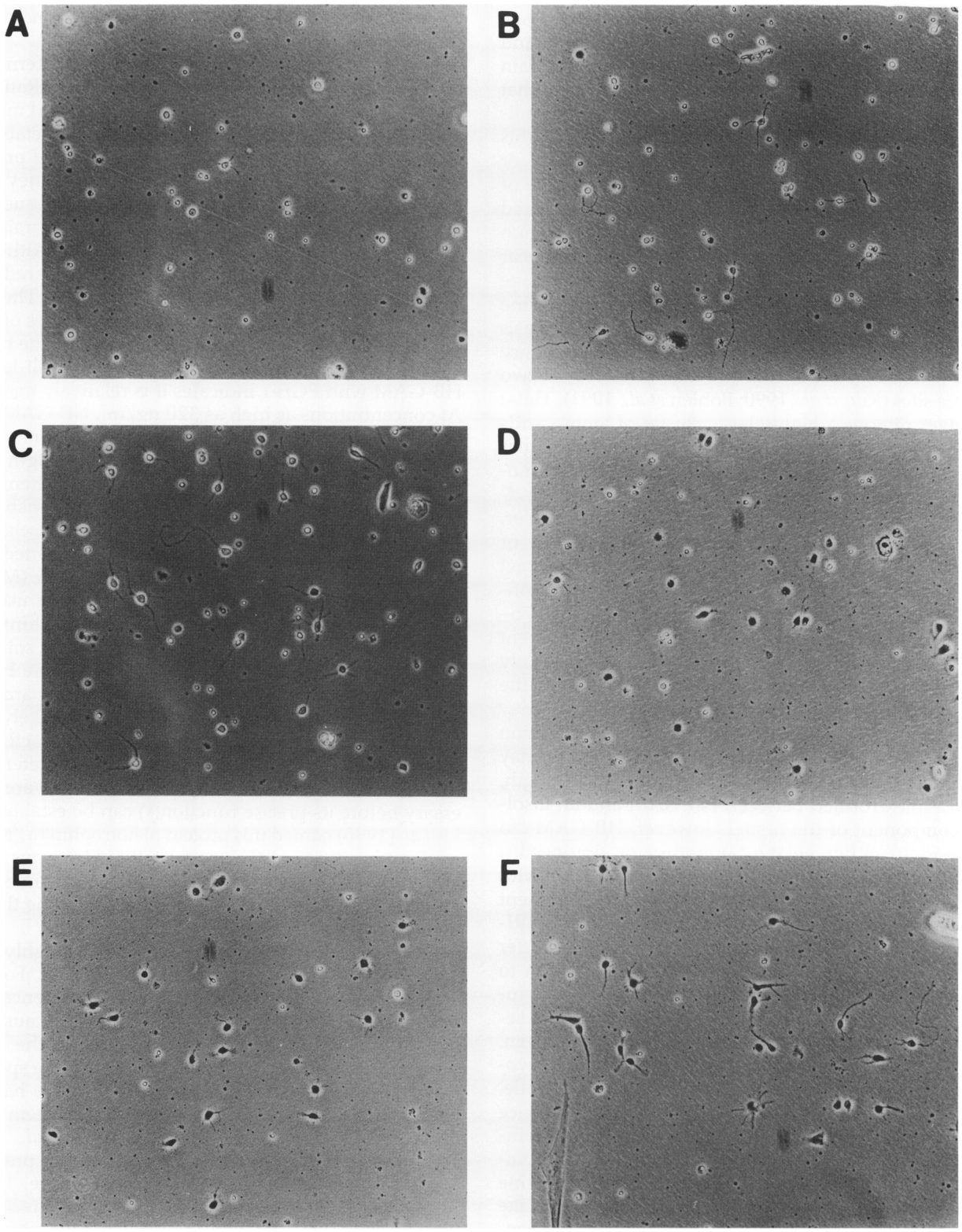


**Figure 5.** Photomicrographs of the neurite extension assay. The photomicrographs were taken at the time the assay was scored with a 20× objective on a Nikon Diaphot inverted phase contrast microscope. A–F are representative fields of the corresponding treatments described in Figure 4.

thermore, these activities may be increased or decreased by the pH of the buffers used during extraction and subsequent purification. We noted that under certain conditions used during the purification of HB-GAM that there was a nearly quantitative conversion of the full-length protein to a truncated form. It appears that this proteolytic activity has a different apparent affinity for heparin-Sepharose than HB-GAM because cleavage of HB-GAM was not observed if a salt gradient was used to elute the heparin-Sepharose rather than batch elution with 1.5 M NaCl. Therefore, HB-GAM does not appear to undergo additional processing other than cleavage of the signal peptide and the mature form of the bovine protein does consist of 136 amino acids. On the basis of two reports that describe the amino acid sequence of truncated HB-GAM, this cleavage can occur at two or three sites (Kuo *et al.*, 1990; Bohlen *et al.*, 1991). These cleavages remove a highly basic cluster of amino acids at the carboxyl-terminus. It was proposed that similar basic clusters of amino acids observed in the amino-terminal region were responsible for the heparin-binding activity of HB-GAM (Kuo *et al.*, 1990). However, we directly compared the heparin-binding activity of the truncated form of HB-GAM with that of the intact protein and found that they are eluted from heparin-Sepharose with the same concentration of NaCl (0.75–1.0 M). Therefore, this would indicate that the apparent affinity of HB-GAM for heparin may not be due solely to the basic clusters of amino acids found both at the amino- and carboxyl-termini of the protein.

The recovery of HB-GAM is increased by the addition of NaCl to the homogenization buffer. This result may be due to the disruption, by the increased ionic strength, of a complex formed between HB-GAM and an insoluble component of the tissue. However, HB-GAM did not bind to either gelatin-Sepharose or fibronectin-Sepharose (data not shown), a characteristic shared with a heparin-binding protein isolated from chick basement membranes (retinoic acid-induced heparin binding [RI-HB]) by extraction with a high ionic strength buffer (Vigny *et al.*, 1989; Urios *et al.*, 1991). In addition to these common characteristics, RI-HB has a similar apparent molecular weight, shares 50% amino acid sequence homology with HB-GAM, and is not a mitogen. RI-HB is widely expressed in early stages of embryogenesis and later becomes restricted to the lens capsule.

HB-GAM also shares a 50% amino acid sequence identity with MK, a protein expressed in mouse teratocarcinoma cells after treatment with retinoic acid (Kadomatsu *et al.*, 1988). Both proteins contain 10 cysteine residues and their relative positions are conserved. Like HB-GAM, MK has been shown to contain a signal peptide sequence and it has been demonstrated that MK is a secreted protein (Tomomura *et al.*, 1990). The tissue distribution of MK is ubiquitous during early embryogenesis beginning at day 5, but becomes more restricted after day 9 until its expression is observed mainly in the kidney (Kadomatsu *et al.*, 1990).

The tissue distribution of HB-GAM was determined by RNA and protein blotting techniques (Rauvala, 1989). In 6-d postnatal rat tissues the HB-GAM mRNA was abundant in brain and virtually undetectable in liver, spleen, kidney, and heart. The HB-GAM protein was detected by western blotting in brain, kidney, and to a lesser extent, in heart tissue. In adult rat tissues the HB-GAM mRNA and protein was detected in brain but not in liver, spleen, kidney, or heart tissues. Although the level of expression of HB-GAM seems to be reduced in adult tissues, it is still relatively abundant. The evidence presented here revealed higher quantities of HB-GAM in chicken heart than was found in bovine brain.

Comparison of the mitogenic activity of full-length HB-GAM with FGF-1 indicates it is relatively inactive. At concentrations as high as 320 ng/ml, HB-GAM was unable to stimulate DNA synthesis or proliferation of four different cell strains. However, full-length HB-GAM is active in a neurite extension assay. When used as an attachment substrate, HB-GAM is capable of stimulating near maximal neurite outgrowth in chicken embryo neurons within 42 h. It has been reported that the biological activity of HB-GAM is acid labile (Milner *et al.*, 1989). Because the purification scheme used to isolate HB-GAM for these experiments did not involve the use of detergent solubilization or organic solvents and acids such as those used in reversed-phase HPLC and resulted in apparently homogeneous preparations of full-length HB-GAM within 24 h, it is unlikely that our purification methods resulted in an acid-inactivated preparation of HB-GAM. Further studies to determine the biological activities of this protein in vivo are necessary before its precise function(s) can be established. Li *et al.* (1990) named this protein pleiotrophin to "reflect its diverse activities." The name HB-GAM seems more appropriate as it was the name given by Merenmies and Rauvala (1990) who were the first to determine the sequence of the cDNA encoding this protein.

The amino acid sequence of HB-GAM is highly conserved even among nonmammalian species. For example, there are only seven amino acid differences between the human and chicken sequences. Because the structure of HB-GAM has been conserved, the functional properties may be similar among diverse species. Its role as a neurotrophic factor (NTF) in vivo has not been established. The suggestion that this protein is indeed an NTF may be premature (Kovesdi *et al.*, 1990; Bohlen *et al.*, 1991). The following set of criteria provides a basis for the definition of an NTF (Walicke, 1989). These criteria are 1) a demonstration of specific receptors on neurons, 2) available in the normal neuronal environment, and 3) NTF effects in vivo as well as in vitro. Certainly the second criteria has been satisfied by Ravaula (1989); however, evidence for specific neuronal receptors for HB-GAM is lacking and evidence for in

vivo effects has not yet been established. Laminin and fibronectin both promote neurite extension but neither are considered to be an NTF. HB-GAM appears to function more like these extracellular matrix proteins rather than as a soluble NTF.

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