Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 188-194

CHARACTERIZATION OF HEPARIN-BINDING GROWTH-ASSOCIATED FACTOR RECEPTOR ON NIH 3T3 CELLS

Ming-Der Kuo, Shuan Shian Huang and Jung San Huang

Department of Biochemistry and Molecular Biology
St. Louis University School of Medicine
St. Louis, MO 63104

Received November 6, 1991

Summary -- Scatchard plot analysis of the binding of ^{125}I-labeled heparin binding cell growth-associated factor (^{125}I-HBGAF) to NIH 3T3 cells revealed a single class of high affinity receptors (~5000/cell) with Kd of ~0.6 nM. ^{125}I-HBGAF was covalently cross-linked to the cell surface receptor on NIH 3T3 cells with disuccinimidyl suberate (DSS). Two ^{125}I-HBGAF-cross-linked complexes of 170 kDa and 142 kDa were observed on SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions. The ^{125}I-HBGAF-cross-linked complex formation was completely abolished in the presence of ≥ 100-fold excess of unlabeled HBGAF but not PDGF, EGF, aFGF, bFGF, or insulin. ^{125}I-HBGAF appeared to undergo rapid internalization and relatively slow degradation following binding to the HBGAF receptor on NIH 3T3 cells. These results suggest that NIH 3T3 cells express a high affinity HBGAF receptor which shows two different estimated molecular masses of ~155 kDa and ~127 kDa. This high affinity HBGAF receptor was also found to express in other cell types.

Heparin binding growth-associated factor or molecule (HBGAF or HBGAM), also known as p18 (1-3), pleiotrophin (PTN) (4) or heparin binding neurotrophic factor (HBNF) (5), is a recently discovered neurotrophic and mitogenic factor. HBGAF was found to stimulate the neurite outgrowth of primary rat neurons (1,2,4,5) and cultured pheochromocytoma cells (PC 12) (3). HBGAF was also reported to be a mitogenic factor toward fibroblasts (4). HBGAF antigen and transcript were localized in neurons (1,2). The time period of expression of HBGAF was found to correlate with that of rapid sprouting of axons and dendrites in the brain (1,2). The neuronal localization and developmental regulation of expression suggest a physiological role for HBGAF in the growth and maturation of brain and other tissues (1,2,4).

The primary structure of HBGAF was elucidated by amino acid sequencing (3,5) and by molecular cloning (2,4). The deduced amino acid sequence of HBGAF

0006-291X/92 $1.50

cDNA revealed that HBGAF possesses a classical signal peptide sequence of secretory proteins. The amino acid sequence of HBGAF does not show any homology with nerve growth factor (NGF) (6), fibroblast growth factors (FGFs) (7) or other known mitogenic factors (8,9). However, the predicted amino acid sequence of a retinoic acid-responsive gene, MK, showed ~50% identity including 10 cysteine residues (10). Interestingly, both HBGAF and the MK gene product have been implicated in the regulation of cell differentiation, suggesting a new gene family of developmentally regulated proteins (1,2,10).

As a polypeptide factor, HBGAF exerts its functions through interaction with specific cell surface receptors in target cells (3). To elucidate the molecular mechanism of HBGAF functions, the characterization of the HBGAF receptor is required. The detailed characterization of the HBGAF receptor has not yet been reported. In a previous study (3), we found an HBGAF receptor with Kd of ~8 nM in NIH 3T3 and pheochromocytoma cells (PC 12). This Kd seemed somewhat higher than those reported for other neurotrophic and mitogenic factors for binding to their respective receptors (7-9). It was possible that the acidic conditions employed previously for preparation of HBGAF may have altered the affinity of HBGAF to its receptor (3). We, therefore, decided to further characterize the HBGAF receptor using HBGAF prepared without acidic treatment. Using HBGAF prepared without acidic treatment as ligand, we were able to detect a high affinity receptor for HBGAF in NIH 3T3 cells and other cell types. In this communication, we demonstrate that NIH 3T3 cells show a single class of high affinity receptor which has two different molecular masses of 155 kDa and 127 kDa. We also show that at 37°C, the cell surface-bound ^{125}I-HBGAF undergoes receptor-mediated internalization and degradation.

Materials and Methods

Materials -- Na^{125}I (17 Ci/mg) was purchased from Du Pont-New England Nuclear. Acidic fibroblast growth factor (aFGF), sulfated Sephadex G-50 (Sulfadex) and heparin-Sepharose 4B were prepared as described previously (3). Heparin from porcine intestinal mucosa, bovine insulin, mouse epidermal growth factor (EGF), high molecular mass protein standards, and chloramine T were obtained from Sigma, St. Louis, MO. Recombinant human platelet-derived growth factor (PDGF, c-sis) and basic fibroblast growth factor (bFGF) were obtained from Amgen Biologicals, CA. Disuccinimidyl suberate (DSS) was purchased from Pierce Chemical Company. NIH 3T3 cells and other cell lines obtained from American Type Culture Collection were grown at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum.

Isolation of Bovine HBGAF and Iodination of HBGAF -- The purification procedure employed to isolate HBGAF from bovine brain was essentially identical with that previously reported (3) except that during ammonium sulfate fractionation the pH 4.5 adjustment was omitted and the reverse phase HPLC in the last step was replaced by a Mono S FPLC. The 2 M NaCl eluents from heparin-Sepharose 4B were concentrated by ultrafiltration and then dialyzed against 20 mM sodium phosphate buffer pH 6.0 containing 0.5 M NaCl. HBGAF was eluted at 0.7 M NaCl from the Mono S column of FPLC with a linear gradient of NaCl from 0.5 to 1.0 M. The

HBGAF purified from the Mono S FPLC appeared to be homogeneous based on the appearance of a single band on 15% SDS-polyacrylamide gel electrophoresis.

Iodination of HBGAF -- Four micrograms (4 μg) of HBGAF were iodinated with 2 mCi of $Na^{125}I$ and 5 μg chloramine T in 150 μl of 200 mM sodium phosphate buffer, pH 7.2. After 90 sec at 20°C, the reaction was terminated by sequential addition of 50 μl of 2 mg/ml sodium metabisulfite and 50 μl of 10 mM potassium iodide. ^{125}I-HBGAF and free ^{125}I were separated on Sephadex G-25 in 50 mM sodium phosphate buffer pH 7.2 and 0.1% bovine serum albumin. The specific radioactivity of ^{125}I-HBGAF was ~3 x 10^5 cpm/ng.

Binding Assay and Cross-linking of the ^{125}I-HBGAF-HBGAF Receptor Complex -- Cells were grown to confluence in Costar 24 well cluster dishes in DMEM containing 10% fetal calf serum. The binding medium (0.15 ml) contained ^{125}I-HBGAF in 5 mM HEPES, pH 7.4, 0.128 M NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. Binding was performed at 0°C for 3 h. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled HBGAF. More than 90% of ^{125}I-HBGAF specifically bound was removable with 0.2 M acetic acid, 0.5 M NaCl (pH 3.0), suggesting that most of the cell-associated ^{125}I-HBGAF at 0°C is located at the cell surface. The ^{125}I-HBGAF-HBGAF receptor complexes were cross-linked by 0.07 mM DSS as previously described (11)

Results and Discussion

We previously reported a procedure for purification of HBGAF from bovine brain involving ammonium sulfate fractionation, Sulfadex (sulfated Sephadex G-50) column chromatography, heparin-Sepharose 4B column chromatography and reverse phase HPLC (3). In this purification procedure, the ammonium sulfate fractionation and reverse phase HPLC were carried out under acidic conditions, pH 4.5 and 0.1% trifluoroacetic acid, respectively. In order to purify HBGAF without undergoing acidic treatment, we modified this purification procedure by omitting the pH 4.5 adjustment during ammonium sulfate fractionation and replacing the reverse phase HPLC by Mono S FPLC at pH 6.0. This modified procedure permitted us to obtain a homogeneous preparation of HBGAF which showed high affinity to the HBGAF receptor.

For characterization of the HBGAF receptor, NIH 3T3 cells were chosen due to their high expression of the HBGAF receptor. NIH 3T3 cells were incubated with various concentrations of ^{125}I-labeled HBGAF (^{125}I-HBGAF) in the presence of 100-fold excess of unlabeled HBGAF. After 3 h at 0°C, the specific binding of ^{125}I-HBGAF was determined. As shown in Fig. 1, ^{125}I-HBGAF bound to the NIH 3T3 cells in a dose-dependent manner. Scatchard plot analysis of the binding data revealed a single class of high affinity receptors (~5,000 receptors/cell) with an apparent dissociation constant (Kd) of ~0.6 nM (Fig. 1, inset). This apparent Kd appeared to be much lower than that previously determined (3). Possibly the acidic treatment employed previously to purify the ligand HBGAF altered the affinity of HBGAF to HBGAF receptor (3). To identify the molecular weight of the HBGAF receptor, cross-linking experiments were performed. The ^{125}I-HBGAF-HBGAF receptor complex was cross-linked with 0.07 mM DSS and analyzed

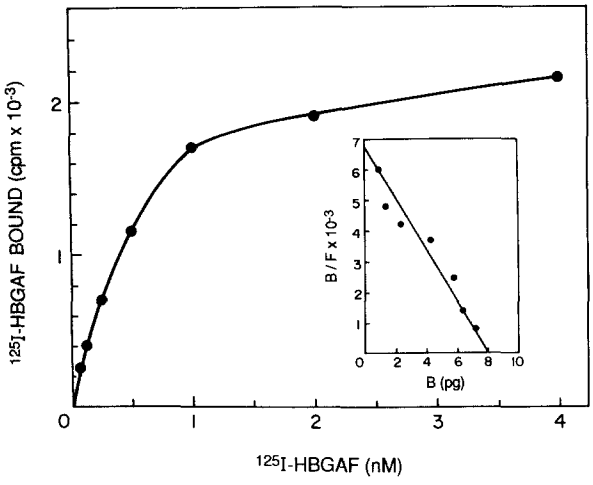


Fig. 1. Binding of ^{125}I-HBGAF to NIH 3T3 Cells.
Monolayers of NIH 3T3 cells in 24-well cluster plates were incubated with various concentrations (0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 nM) of ^{125}I-HBGAF in the presence and absence of 100-fold excess of unlabeled HBGAF. After 3 h at 0°C, the cells were washed and cell-associated ^{125}I-HBGAF was measured. The assays were carried out in duplicate. The specific binding of ^{125}I-HBGAF to NIH 3T3 cells was determined by subtraction of the nonspecific binding from the total binding. The nonspecific binding represented a maximum of 60% of the total binding. Scatchard plot analysis of the binding data was then performed, and a straight line was obtained using a linear least-squares regression program (inset).

by 7.5% SDS-polyacrylamide gel electrophoresis following incubation of NIH 3T3 cells with 0.5 nM ^{125}I-HBGAF in the presence of various concentrations of unlabeled HBGAF. Two ^{125}I-HBGAF-HBGAF receptor complexes of 170 kDa and 142 kDa were observed on 7.5% SDS-polyacrylamide gel electrophoresis under either reducing (Fig. 2A) or non-reducing conditions (data not shown). The radioactivity observed at the top of the gel was possibly an aggregated form of the ^{125}I-HBGAF-HBGAF receptor complex which was unable to enter 7.5% and 5% SDS-polyacrylamide gels. The specificity of the ^{125}I-HBGAF-HBGAF receptor complex formation is evidenced by the observations that the presence of 100-fold excess of unlabeled HBGAF completely abolished the formation of the ^{125}I-HBGAF-HBGAF receptor complexes (Fig. 2A) and that other known mitogenic factors (PDGF, aFGF, EGF and insulin) failed to have any significant effect on the formation of the ^{125}I-HBGAF complexes (Fig. 2B). bFGF was also found not to have an effect on the complex formation (data not shown). Based on the molecular mass of HBGAF (~15 kDa), the molecular masses of the two species of the HBGAF receptor were estimated to be 155 kDa and 127 kDa. The relationship of these two size HBGAF receptors is not yet clear. Possibly, the 127-kDa HBGAF is a proteolytic product of the 155-kDa HBGAF receptor. Alternatively, the 155-kDa and 127-kDa HBGAF receptors are products of two different genes or products of alternative splicing of a single gene.

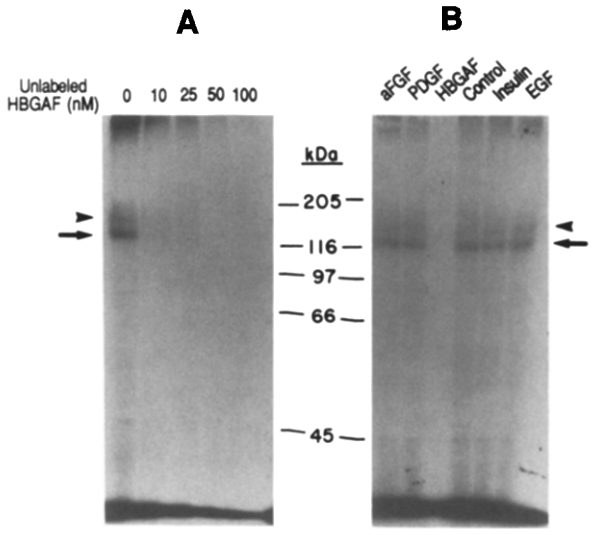
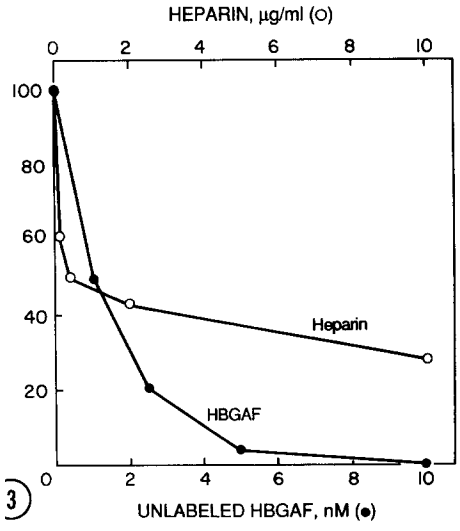


Fig. 2. Cross-linking of ^{125}I-HBGAF-HBGAF Receptor Complexes on NIH 3T3 Cells. Monolayers of NIH 3T3 cells grown on 35-mm Petri dishes were incubated with 0.5 nM of ^{125}I-HBGAF in the presence of various concentrations of unlabeled HBGAF (A) or 100-fold excess of unlabeled HBGAF, PDGF, aFGF, EGF or insulin and control (without factors) (B). After 3 h at 0°C, the cells were washed and cross-linked with 0.07 mM DSS. The ^{125}I-HBGAF-HBGAF receptor cross-linked complexes were then analyzed by 7.5% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The arrowhead and arrow indicate the locations of 170-kDa and 142-kDa ^{125}I-HBGAF-HBGAF receptor complexes, respectively.

HBGAF exhibits high affinity to heparin-Sepharose (1-5). It would be of interest to investigate the effect of various concentrations of heparin on the ^{125}I-HBGAF binding to the HBGAF receptor. As shown in Fig. 3, heparin inhibited the ^{125}I-HBGAF binding to the HBGAF receptor in a dose-dependent manner with a half maximal concentration (ID_{50}) of 0.4 μg/ml. By comparison, unlabeled HBGAF showed a 50% inhibition at ~1 nM concentration which is close to the Kd of HBGAF (Fig. 3). These results suggest that heparin is a potent inhibitor for the receptor binding activity of HBGAF. The mechanism of inhibition by heparin is unknown. It is possible that heparin inhibits the binding of ^{125}I-HBGAF to the HBGAF receptor through interaction with ^{125}I-HBGAF.

The binding of epidermal growth factor (EGF) to the EGF receptor results in activation of its cytoplasmic protein tyrosine kinase domain, followed by internalization and degradation of both EGF receptor and EGF (12). This process is referred to as receptor-mediated endocytosis and appears to be a typical biochemical character of many growth factor receptors (7-9). To determine whether the HBGAF receptor mediated the endocytosis of cell surface-bound ^{125}I-HBGAF, we investigated the internalization and degradation of ^{125}I-HBGAF in NIH 3T3 cells. In this experiment, NIH 3T3 cells were incubated with 0.5 nM of ^{125}I-HBGAF in the presence and absence of 100-fold excess of unlabeled HBGAF.



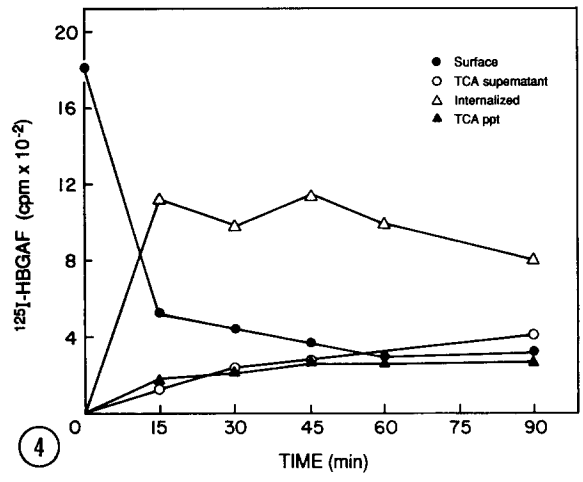


Fig. 3. Effect of Heparin on ^{125}I-HBGAF Binding to NIH 3T3 Cells.
Monolayers of NIH 3T3 cells (24-well cluster plates) were incubated with 0.5 nM of ^{125}I-HBGAF in the presence of various concentrations of heparin or unlabeled HBGAF. After 3 h at 0°C, the specific binding of ^{125}I-HBGAF to the cells was determined. The specific binding of ^{125}I-HBGAF in the absence of heparin (1632 cpm) was taken as 100% binding.

Fig. 4. Internalization and Degradation of Cell Surface-bound ^{125}I-HBGAF in NIH 3T3 Cells.
^{125}I-HBGAF (0.5 nM) was incubated with NIH 3T3 cells (in 24-well cluster plates) in the presence and absence of 100-fold excess of unlabeled HBGAF in DMEM containing 0.1% bovine serum albumin. The presence of 100-fold excess of unlabeled HBGAF in the control experiment provided an estimation of nonreceptor-mediated binding or processes. After 3 h at 0°C, the cells were washed and then incubated at 37°C with DMEM containing 0.1% bovine serum albumin for different time periods. At each time period, the cell surface-bound ^{125}I-HBGAF, internalized ^{125}I-HBGAF, ^{125}I-HBGAF degraded and released to the medium and ^{125}I-HBGAF dissociated from the cells were determined. The pH 3.0/0.5 M NaCl wash of cells was employed to estimate the cell surface-bound ^{125}I-HBGAF and internalized ^{125}I-HBGAF. More than 90% of cell-associated ^{125}I-HBGAF was pH 3.0/0.5 M NaCl-removable after incubation of cells with ^{125}I-HBGAF at 0°C for 3 h. Ten per cent trichloroacetic acid (TCA) treatment of the media was used to determine ^{125}I-HBGAF dissociate from the cells (TCA precipitate) and ^{125}I-HBGAF degraded and released into the medium (TCA supernatant). The ^{125}I-HBGAF degraded and released into the medium was identified mainly as ^{125}I-iodotyrosine according to a published procedure (13).

After 3 h at 0°C, the cells were washed and warmed up to 37°C. The cell surface-bound ^{125}I-HBGAF, internalized ^{125}I-HBGAF and ^{125}I-HBGAF released into the medium were then measured over a time period of 90 min. As shown in Fig. 4, upon warming at 37°C, a small fraction (~13%) of cell surface-bound ^{125}I-HBGAF dissociated from the cell (TCA precipitate) and reached a maximum at 30 min incubation, whereas the majority of cell surface-bound ^{125}I-HBGAF underwent internalization. Interestingly the internalized ^{125}I-HBGAF appeared to be relatively stable. Only a small fraction (~20%) of it was degraded and released as ^{125}I-iodotyrosine into the medium (TCA supernatant) even after 90 min incubation. The mechanism of internalized ^{125}I-HBGAF stability is not known. It is possible that the high content of disulfide bonds (five disulfide bonds

per molecule of HBGAF) (3) may explain its relative stability to proteolytic digestion. Furthermore, the heparin-binding properties of HBGAF may also contribute to its stability in endocytic vesicles and/or lysosomes. The potential complex formation of HBGAF with proteoglycans in the lumen of these intracellular organelles may result in its inaccessibility to proteolytic enzymes. Basic fibroblast growth factor (bFGF), also a high affinity heparin-binding protein, has been shown to be relatively stable after internalization with its receptor (14).

The distribution of the HBGAF receptor in different cell types has not been studied. For this reason, we examined the expression of the HBGAF receptor in normal rat kidney (NRK) cells, human mammary adenocarcinoma cells (SK-BR-3), pheochromocytoma cells (PC 12), human epidermoid carcinoma cells (A431), human hepatocarcinoma cells (HepG2) and mouse neuroblastoma cells (NB41A3) by measuring the specific binding of ^{125}I-HBGAF to these cells. Surprisingly, all of these cells expressed the high affinity HBGAF receptor. The numbers of the HBGAF receptor expressed in these cells varied from a few hundred to a few thousand receptors per cell. The functional significance of the HBGAF receptor in these cells is not known and warrants investigation.

Acknowledgments -- We thank Drs. William S. Sly and Pauline O'Grady for reviewing the manuscript and Maggie Klevorn for preparing the manuscript.

References

1. Rauvala, H. (1989) *EMBO J.* **8**, 2933-2941.
2. Merenmies, J., and Rauvala, H. (1990) *J. Biol. Chem.* **265**, 16721-16724.
3. Kuo, M.-D., Oda, Y., Huang, J.S., and Huang, S.S. (1990) *J. Biol. Chem.* **265**, 18749-18752.
4. Li, Y.-S., Milner, P.G., Chouhan, A.K., Watson, M.S., Hoffman, R.M., Kodner, C.M., Milbrandt, J., and Deuel, T.F. (1990) *Science* **250**, 1690-1693.
5. Böhlen, P., Müller, T., Gautschi-Sova, P., Albrecht, U., Rasool, C.G., Decker, M., Seddon, A., Fafeur, V., Kovesdi, I., and Kretschmer, P. (1991) *Growth Factors* 4, 97-107.
6. Angeletti, R.H., and Bradshaw, R.A. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2417-2420.
7. Gimenez-Gallego, G., Rodkey, J., Bennett, C., Rios-Candelore, M., DiSalvo, J., and Thomas, K. (1985) *Science* **85**, 1385-1388
8. Deuel, T.F. (1987) *Ann. Rev. Cell Biol.* **3**, 443-492.
9. Heldin, C.-H., and Westermark, B. (1989) *Eur. J. Biochem.* **184**, 487-496.
10. Tomomura, M., Kadomatsu, K., Matsubara, S., and Muramatsu, T. (1990) *J. Biol. Chem.* **265**, 10765-10770.
11. O'Grady, P., Kuo, M.-D., Baldassare, J.J., Huang, S.S., and Huang, J.S. (1991) *J. Biol. Chem.* **266**, 8583-8589.
12. Aharonov, A., Pruss, R.M., and Hershman, H.R. (1978) *J. Biol. Chem.* **253**, 3970-3977.
13. Huang, J.S., Huang, S.S., and Kuo, M.-D. (1986) *J. Biol. Chem.* **261**, 11600-11607.
14. Moscatelli, D. (1988) *J. Cell Biol.* **107**, 753-759.