Vol. 106, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1982 Pages 202-209

RECOMBINANT DNA-DERIVED METHIONYL HUMAN GROWTH HORMONE IS SIMILAR IN MEMBRANE BINDING PROPERTIES TO HUMAN PITUITARY GROWTH HORMONE

Ron G. Rosenfeld, Bharat B. Aggarwal,
Raymond L. Hintz and Laura A. Dollar

Department of Pediatrics
Stanford University Medical Center
Stanford, California 94305

and

Department of Protein Biochemistry
Genentech, Inc.
South San Francisco, California 94080

Received April 5, 1982

N-terminal methionyl human growth hormone (met-hGH), expressed in Escherechia coli by recombinant DNA techniques, was compared to native human pituitary growth hormone in receptor-binding activity. Met-hGH was indistinguishable from pituitary hGH in binding to specific hGH receptors on cultured IM-9 lymphocytes and to "lactogenic" receptors on pregnant rat liver membranes. Additionally, met-hGH and pituitary hGH were equivalent in ability to induce hGH receptor loss in IM-9 cells, with an 80% decrease in membrane binding sites following 20 hour preincubation with 10 ng/ml of either met-hGH or pituitary hGH. We conclude that the receptor-binding activity of bacterially synthesized met-hGH and pituitary hGH are identical, suggesting a common tertiary structure.

INTRODUCTION

Since non-primate growth hormone has little growth-stimulatory action in humans, definitive treatment of growth hormone deficiency has necessitated the administration of hGH extracted from human cadaver pituitary glands (1). The typical patient requires 20-50 cadaver pituitaries per year of therapy, and the obvious limitations upon this supply have restricted both the dosages employed and the spectrum of clinical disorders which can be treated with current hGH preparations.

The expression of methionyl-hGH in Escherechia coli, using recombinant DNA techniques, and the capability of purifying the cloned protein to homo-

Abbreviations used are: HEPES, N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo) tetraacetic acid; Tris, tris (hydroxymethyl) aminomethane.

0006-291X/82/090202-08$01.00/0

geneity, have provided a promising and virtually limitless new source of hGH (2,3). By radioimmunoassay, pituitary hGH and bacterially synthesized met-hGH have equivalent specific activities. However, the biological action of hGH, like that of other protein hormones, is presumably mediated through binding to specific receptors on the plasma membranes of target cells, and receptorassays based upon such interactions are felt to provide a better measure of biological activity (4-6). The following study was designed to evaluate the ability of bacterially synthesized N-methionyl-hGH to compete for occupancy of specific hGH receptors on cultured lymphocytes and of "lactogenic" receptors on pregnant rat liver membranes. Additionally, N-methionyl-hGH and pituitary hGH were compared in their ability to induce loss of homologous receptors on IM-9 lymphocytes. The results to be described indicate that pituitary hGH and bacterially synthesized N-methionyl-hGH are characterized by identical membrane binding behavior, suggesting that both proteins share a common tertiary structure.

MATERIALS AND METHODS

Hormone Source: Human growth hormone, derived from cadaver pituitary glands, was kindly provided by the National Pituitary Agency. Human prolactin was a gift from the National Institutes of Health. Two lots of bacterially derived N-terminal methionyl-hGH were employed in these studies, and were designated met-hGH-I and met-hGH-II. These bacterially synthesized proteins were purified from Escherechia coli extract and characterized as previously reported (3). Both preparations exhibited a single band on both sodium dodecyl sulfate and native polyacrylamide gel electrophoresis. Met-hGH-I demonstrated no pyrogenic activity, while met-hGH-II showed the presence of pyrogenic activity, as measured by the limulus assay (7).

[^{125}I] -hGH was prepared from pituitary hGH by a modification of the method of Hunter and Greenwood (8). Specific activity ranged from 90-100 uCi/ug. Before all assays, the iodinated peptide was repurified by gel filtration on a Sephadex G-50 column (1.5 x 90 cm) at 4^{o}C, using 100 mM HEPES buffer (pH 7.0), with 1% bovine serum albumin, 120 mM NaCl, 1.2 mM MgSO4, 2.5 mM KCl, 15 mM Na acetate, 10 mM dextrose, and 1 mM EDTA.

IM-9 cells: IM-9 cells, an established line of human lymphocytes, were grown in continuous culture in RPMI-1640 medium with 25 mM HEPES buffer (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 10% fetal bovine serum (Grand Island Biological Co.), 4 mM L-glutamine (Grand Island Biological Co.), penicillin (100 U/ml), and streptomycin (100 ug/ml). The cells were grown in 250-ml Falcon flasks (Falcon Plastics, Los Angeles, CA) at 37^{o}C. At 72 hours, the cells were divided 1 to 3, and fresh medium was added to each flask.

IM-9 radioreceptorassay: Radioreceptorassay of hGH was performed by a modification of the method of Lesniak et al (5). IM-9 cells (20 x 10^{6} cells/ml) were incubated for 100 minutes at 30^{o}C with [^{125}I] -hGH and increasing con-

centrations of unlabeled pituitary hGH or met-hGH in a final volume of 500 ul. At the end of the incubation duplicate 200 ul aliquots were layered over 150 ul of HEPES buffer in plastic microtubes at 4°C. Samples were then centrifuged, supernatants discarded, and pellets counted for 10 minutes for determination of cell-bound radioactivity. Nonspecific binding, defined as the counts remaining bound in the presence of 100 ug/ml of hGH, was subtracted from all data points.

IM-9 receptor modulation assay: The ability of the hGH peptides to both occupy and down-regulate the IM-9 receptor was determined by a modification of the method of Rosenfeld and Hintz (6). In this noncompetitive radioassay, IM-9 cells were preincubated with unlabeled hGH for 20 hours at 37°C, washed extensively, and residual binding sites determined by measuring the ability of preincubated cells to subsequently bind [^{125}I]-hGH, as described above. All preincubation flasks were run in duplicate, and nonspecific binding was subtracted from all data points.

Rat liver membrane radioreceptorassay: Livers from pregnant Sprague-Dawley rats were homogenized in 0.25M ice-cold sucrose, and crude membrane preparations were obtained by sequential ultracentrifugation, according to the method of Cuatrecasas (9). The plasma membrane fraction sedimenting between 12,000x g and 40,000x g was resusupended in 0.05 M Tris-HCl buffer, pH 7.4, containing 1% bovine serum albumin. Membrane preparations were incubated for 20 hours at 4°C with [^{125}I] -hGH and increasing concentrations of unlabeled pituitary hGH, met-hGH or human prolactin, in a final volume of 500 ul. Following incubation, 1 ml of Tris-HCl buffer was added to each tube, samples were centrifuged at 12,000x g for 30 minutes at 4°C, and pellets were counted for determination of membrane-bound radioactivity.

RESULTS

The competition curves of each of the three hGH preparations for the IM-9 hGH receptor, using [^{125}I] -hGH as radioligand, are depicted in Figure I. The

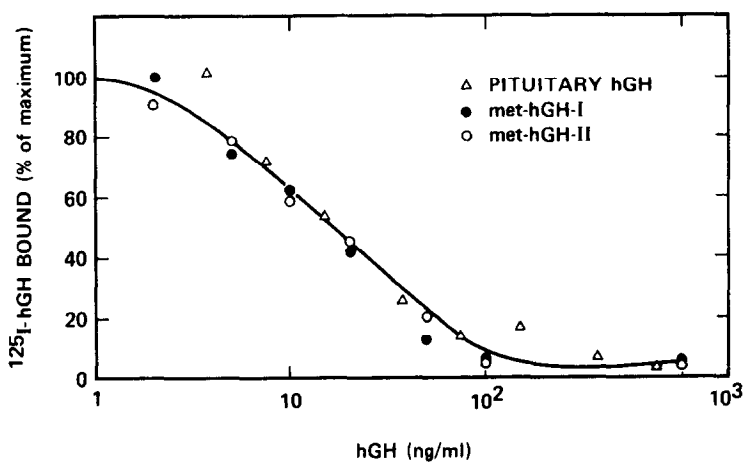

Figure 1: Percent of maximal specific binding of [^{125}I] -hGH to IM-9 cells as a function of the unlabeled hGH concentration. IM-9 cells (20×10^6 cell/ml) were incubated for 100 minutes at 30°C with [^{125}I] -hGH and increasing concentrations of unlabeled pituitary hGH or met-hGH. Non-specific binding has been subtracted from all data points. Specific binding in the absence of unlabeled hormone (Bo) was 8.46%.

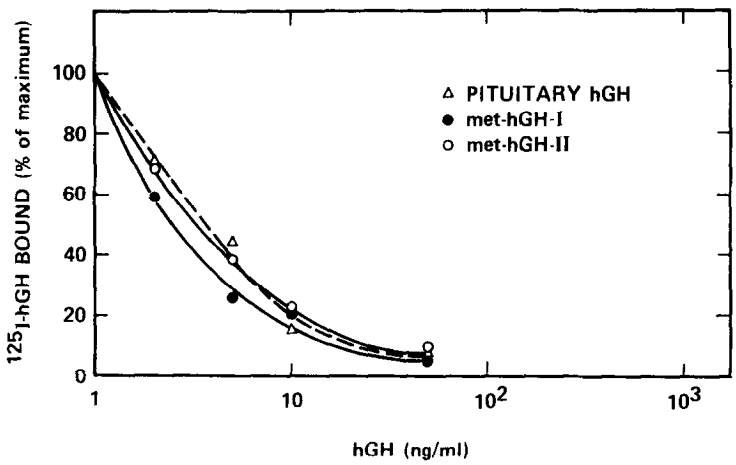

Figure 2: Percent of maximal specific binding of [^{125}I] -hGH to IM-9 cells as a function of the unlabeled hGH concentration during preincubation. IM-9 cells were preincubated for 20 minutes at 37°C with unlabeled pituitary hGH or met-hGH. After extensive washing, the cells were incubated for 100 minutes at 30°C with [^{125}I] -hGH. Specific binding was compared to cells preincubated in the absence of unlabeled hGH.

three proteins demonstrated identical affinities for this receptor, with 50% displacement of [^{125}I] -hGH observed at protein concentrations of 15-17 ng/ml. The hGH-induced loss of homologous binding sites on IM-9 lymphocytes is shown in Figure 2. A 30-40% decrease in binding of [^{125}I] -hGH was observed following preincubation of cells with hormone at concentrations as low as 2 ng/ml, and 80% receptor loss was observed following preincubation with 10 ng/ml of either met-hGH or pituitary hGH. These results are similar to previous reports of hGH-induced down-regulation of homologous receptors, and no significant differences were observed between the three hGH preparations employed here (6,10).

Figure 3 depicts competition for binding to receptors on rat liver membrane preparations. The "lactogenic" nature of this receptor is supported by the relative potency of human prolactin, which was approximately 1/3 as potent as hGH (50% displacement of [^{125}I] -hGH was observed at human prolactin concentrations of 60-70 ng/ml). Fifty percent occupancy of this receptor occurred at pituitary hGH concentrations of 16 ng/ml, and met-hGH concentrations of 16 and 20 ng/ml. Again, no significant differences were observed among the three hGH preparations, and the binding characteristics were similar to those previously described for pituitary hGH (11).

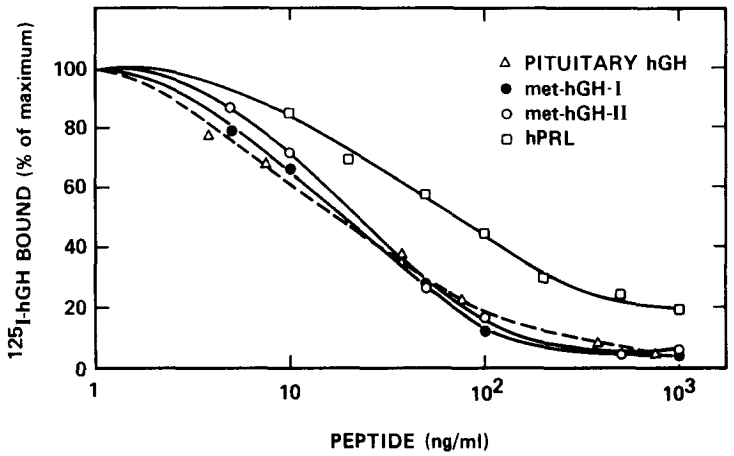

Figure 3: Percent of mximal specific binding of [^{125}I] -hGH to pregnant rat liver membranes as a function of the unlabeled hormone concentration. Membrane preparations were incubated for 20 hours at 4°C with [^{125}I] -hGH and increasing concentrations of unlabeled pituitary hGH, met-hGH or human prolactin. Nonspecific binding has been subtracted from all data points. Specific binding in the absence of unlabeled hormone (Bo) was 21.3%.

DISCUSSION

The interaction between a protein hormone and its cell membrane receptor is the initial step in the expression of the biological activity of that hormone (12). This reaction is critically dependent upon both the tertiary structure of the protein and the integrity of the biologically active binding sites(s) on the surface of the protein. Structural abnormalities in the receptor-binding site of a protein can impair biological activity by altering the affinity of the protein for its receptor. Given et al (13), for example, have reported a structurally abnormal insulin molecule in a patient with diabetes, and have demonstrated that insulin from this patient's serum bound to IM-9 lymphocytes and rat adipocytes only 40% as well as insulin standards. Similarly, Kowarski et al (14) and Rudman et al (15) have described patients with growth failure, normal immunoreactive hGH, but decreased radioreceptorassayable serum hGH levels, and have postulated the presence of a biologically inactive hGH.

Our data indicate that met-hGH produced by Escherechia coli is equivalent to pituitary-derived hGH in its ability to compete for occupancy of hGH membrane receptors. The IM-9 lymphocyte has been shown to possess receptors which are highly specific for hGH. Non-primate growth hormones do not com-

pete with [^{125}I] -hGH for this receptor, and both human prolactin and human placental lactogen are less than 1% as potent as hGH (16). The two met-hGH preparations proved to be identical to pituitary-hGH in their ability to compete with [^{125}I] -hGH for this highly specific receptor. Binding of hGH to pregnant rat liver membranes, on the other hand, may well be to receptors with different specifities than that seen on the IM-9 cell, since these "lactogenic" receptors are competed for by both human and rat prolactin, as well as by hGH (11,17). In our study, human prolactin was approximately 40% as potent as pituitary hGH in competing with [^{125}I] -hGH for liver membrane receptors. Again, however, both met-hGH preparations were equivalent to pituitary hGH in their affinity for this receptor.

The bacterial hGH possesses an additional methionine residue at the N-terminal. Our data indicate that this residue, which arises from the AUG start codon inserted at the beginning of the hGH gene, does not alter the membrane binding characteristics of hGH. This is further supported by the similar abilities of met-hGH and pituitary hGH to not only occupy the IM-9 hGH receptor, but to induce homologous receptor loss. This down-regulation of hGH receptors has been linked to internalization of the ligand-receptor complex and degradation of the protein, although its role in mediating hGH's biological actions remains uncertain (18,19). Since the actual target for hGH action has not yet been identified, the IM-9 membrane binding site may only be termed a receptor because of its ability to selectively recognize hGH. However, by analogy with other protein hormones, the affinity of a ligand for its receptor is proportional to that ligand's ability to exert a specific biological action under both in vitro and in vivo conditions (12). This has been most convincingly demonstrated for insulin and insulin analogues, and it is of note that Keefer et al (20) have recently demonstrated that human insulin prepared by recombinant DNA techniques not only exhibits similar affinity for the insulin receptor, when compared with native human insulin, but is equipotent in stimulating lipogenesis in isolated rat fat cells.

The characterization of the receptor-binding activity of met-hGH reported here agrees with recent animal and human studies with bacterially-derived hGH. Olson et al (3) have shown that met-hGH promotes weight gain and tibial growth in hypophysectomized rats, and we have recently demonstrated that met-hGH and pituitary hGH are similar in their ability to stimulate somatomedin production and insulin resistance in normal adults (21,22). The combination of in vitro and clinical studies confirms the integrity of the tertiary structure of bacterially-produced met-hGH, and supports the potential usefulness of recombinant DNA-derived hGH in the treatment of disorders of growth.

REFERENCES

1. Tanner, J.M., Whitehouse, R.H., Hughes,P.C.R. and Vince, F.P. (1971) Arch. Dis. Child. 46, 745-782.
2. Goedell, D.V., Heynecker, H.L., Hozumi, T., Arentzen, R., Itakura, K., Yansura, D.G., Ross, M.J., Miozzari, G., Crea, R. and Seeburg, P.H. (1979) Nature 281, 544-548.
3. Olson, K.D., Fenno, J., Lin, N., Harkins, R.N., Snider, C., Kohr, W.H., Ross, M.J., Fodge, D., Prender, G. and Stebbing, N. (1981) Nature 293, 408-411.
4. Tsushima, T. and Friesen, H.G. (1973) J. Clin. Endocrinol. Metab. 37, 334-337.
5. Lesniak, M.A., Roth, J., Gorden, P. and Gavin, J.R. (1973) Nature 241, 20-22.
6. Rosenfeld, R.G. and Hintz, R.L. (1980) J. Clin. Endocrinol. Metab. 50, 62-69.
7. Dinarello, C.A. (1981) in Methods for Studying Mononuclear Phagocytosis ed. Adams, D.O. (Academic Press, New York, N.Y.).
8. Hunter, W.M. and Greenwood, F.C. (1962) Nature 194, 495-496.
9. Cuatrecasas, P. (1972) Proc. Natl. Acad. Sci. USA 69, 318-322.
10. Lesniak, M.A. and Roth, J. (1976) J. Biol. Chem. 251, 3720-3729.
11. Posner, B.I., Kelly, P.A., Shiu, R.P.C. and Friesen, H.G. (1974) Endocrinology 95, 521-531.
12. Kahn, C.R. (1976) J. Cell Biol. 70, 261-286.
13. Given, B.D., Mako, M.E., Tager, H.S., Baldwin, D., Markese, J., Rubenstein, A.H., Olefsky, J., Kobayaski, M., Kolterman, O. and Poucher, R. (1980) N. Engl. J. Med. 302, 129-135.
14. Kowarski, A.A., Schneider, J., Ben-Galim, E., Weldon, V.V. and Daughaday, W.H. (1978) J. Clin. Endocrinol. Metab. 47, 461-464.
15. Rudman, D., Kutner, M.H., Blackston, R.D., Cushman, R.A., Bain, R.P. and Patterson, J.H. (1981) N. Engl. J. Med. 305, 123-131.
16. Lesniak, M., Gorden, P. and Roth, J. (1977) J. Clin. Endocrinol. Metab. 44, 838-849.
17. Posner, B.I., Kelly, P.A. and Friesen, H.G. (1974) Proc. Natl. Acad. Sci. USA, 71, 2407-2410.
18. Rosenfeld, R.G. and Hintz, R.L. (1980) J. Clin. Endocrinol. Metab. 51, 368-375.
19. Hizuka, N., Gorden, P., Lesniak, M.A., Van Obberghen, E., Carpenter, J.L. and Orci, L. (1981) J. Biol. Chem. 256, 4591-4597.

20. Keefer, L.M., Piron, M.A. and De Meyts, P. (1981) Proc. Natl. Acad. Sci. USA 78, 1391-1395.
21. Rosenfeld, R.G., Wilson, D.M., Dollar, L.A., Bennett, A. and Hintz, R.L. (1982) J. Clin. Endocrinol. Metab. (in press).
22. Hintz, R.L., Rosenfeld, R., Wilson, D. and Bennett, A. (1981) Abstracts of the First Joint Meeting of The Lawson Wilkins Pediatric Endocrine Society - European Society of Pediatric Endocrinology, p. 21.