Alteration in the Receptor Binding Specificity of Human Growth Hormone by Genomic Exon Exchange

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The biological activities of the GH-PRL family of hormones are mediated by selective binding to two classes of cell membrane receptors, somatogen and lactogen. Primate GH such as human GH (hGH) are unusual in that they bind to both classes of receptors. Replacement of exons 3 or 4 of the hGH gene by the corresponding exons of the rat PRL or rat GH genes results in the synthesis of chimeric proteins which retain the ability to bind to lactogen receptors but can no longer bind to somatogen receptors. This selective loss of somatogen receptor binding in the chimeric proteins suggests that certain of the structural determinants of somatogen and lactogen receptor binding activities in hGH are distinct and can be separately modified by a limited number of amino acid substitutions. (Molecular Endocrinology 4: 101–107, 1990)

INTRODUCTION

The human GH (hGH)-PRL gene family includes a cluster of GH-related genes on chromosome 17: the normal pituitary GH (hGH-N), a GH variant expressed by the placenta (hGH-V), two chorionic somatomammotropin genes (hCS-A and hCS-B), and one presumed pseudogene (hCS-L) (1, 2). The more structurally divergent PRL gene is encoded on chromosome 6 (3, 4). Each of these genes shares an identical number of exons and the introns interrupt the codons in homologous positions. The biological activities of these hormones are mediated by binding to two classes of membrane-bound receptors on the surface of target cells. The structures of representative members of both the lactogen and somatogen receptors (5, 6) have been established (7–10) although the mode of signal transduction remains unknown. The biological activities elicited by each ligand-receptor interaction are surprisingly divergent considering the structural similarities among both the ligands and the receptors. Lactogen receptor-ligand interactions contribute to the maintenance of lactation, breast development, and normal reproductive function. The effects of somatogen receptor-ligand interactions on linear bone growth and on general metabolic functions are mediated both indirectly through the induction of insulin-like growth factor-1 expression in the liver and by direct effects on peripheral target tissues (11, 12). PRL and CS bind to lactogen receptors, while subprimate GHs bind to somatogen receptors. Human GH-N and hGH-V (and primate GHs in general) are unusual in that they bind to both receptor classes (13; Ray, J., H. Okamura, P. A. Kelly, S. A. Liebhaber, and N. E. Cooke, submitted).

The conservation of exon structure within the GH-PRL gene family is consistent with the hypothesis that these exons may encode conserved functional domains in their respective protein products (14, 15). Recent studies using site-specific mutation and monoclonal antibody binding have mapped three segments within the hGH primary structure which appear to interact with the solubilized external domain of the somatogen receptor (16). Based upon a folded protein model derived from the crystal structure of porcine GH (17), these three regions coalesce into a discrete surface patch. The first region (amino acids 12–19) is encoded by exon 2; the second region (amino acids 56–64) is encoded by exon 3; and the third region (amino acids 167–181) is encoded by exon 5. It is unknown whether these

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same regions are also involved in determining the lactogen receptor binding specificity of hGH or whether this second specificity is structurally distinct. To test the hypothesis that the two binding activities of hGH may have one or more distinct determinants, and that such determinants may be carried on exon encoded domains, we have exchanged corresponding exons between the hGH gene and either the rat PRL (rPRL) or the rat GH (rGH) genes and assayed the resultant chimeric proteins for somatogen and lactogen receptor binding activity.

RESULTS

In previous work we have demonstrated the feasibility of expressing stable chimeric hormones after exon exchange (18). The three exon exchange chimeras studied in the present report were constructed by deleting exon 3 or 4 along with adjacent intron sequences from the hGH gene and replacing each with the corresponding exons of the rPRL (19, 20) or rGH gene (21). A schematic diagram of the three chimeric genes, hGH-rP3, hGH-rP4, and hGH-rG3, and a comparison of the exchanged exon sequences, are shown in Fig. 1, A and B, respectively. Each of these three chimeric genes (as well as hGH) was inserted into a bovine papilloma virus episomal expression vector under the control of the mouse metallothionein (mMT) gene promoter (details in *Materials and Methods*). Mouse fibroblasts were then transfected with each recombinant and stable cell lines were established expressing each of five transfected genes: native hGH, hGH-rP3, hGH-rP4, hGH-rG3, and the expression vector alone (cell line CM2–2). The GH-chimeric proteins secreted into the conditioned media of each line were characterized by Western analysis (Fig. 1C). Each chimeric gene expressed a stable, secreted protein product that was approximately the expected mol wt (hGH-rP3, 21.0 K *vs*. predicted 21.5; hGH-rP4, 23.0 K *vs*. predicted 22.6; hGH-rG3 21.0 K *vs*. predicted 21.9). To further substantiate that correct signal processing was occurring in this system we sequenced the amino terminus of hGH and hGH-rP3 isolated from the corresponding conditioned media. Six cycles of analysis were run (data not shown). The results of these cycles were: Phe-Pro-Thr-Ile-Pro-Leu. This sequence is a perfect match to the first six residues of the mature hGH (data not shown). Therefore, the hGH and hGH-derivative proteins secreted into the media by the four cell lines appeared to represent the predicted products on the basis of immunoreactivity, size, and limited N-terminal sequence analysis.

The conditioned media from each of the five cell lines were assayed for both somatogen and lactogen receptor binding activity. Somatogen receptor binding was measured by specific inhibition of ^{125}I-labeled hGH binding to rabbit liver microsomes (Fig. 2, A and C), while lactogen receptor binding was measured by specific inhibition of ^{125}I-labeled ovine PRL (oPRL) binding to rat liver membranes (Fig. 2, B and D). These two assay systems were selected because they have been very well characterized. In control experiments, rPRL, a pure lactogen, failed to bind to the somatogen receptors (Fig. 2A). Rat GH also failed to significantly displace the ^{125}I-hGH (Fig. 2A) consistent with the documented cross-species incompatibility between rGH and the rabbit somatogen receptor (22). In the lactogen binding assay controls (Fig. 2B), both hGH and rPRL completely displace the ^{125}I-oPRL tracer from the membrane receptors, while rGH showed no competition. These results suggest that protein domains in the chimeric GH proteins encoded by a rPRL gene exon would not specify somatogen receptor binding and that domains encoded by a rGH gene exon would not specify binding to either class of receptor. These useful properties were exploited in the design of the chimeric genes.

The binding inhibition profiles of the three hGH exon exchange derivatives, hGH-rP3, hGH-rP4, and hGH-rG3 are all significantly different than the hGH profile. All three hGH chimeric proteins bind to the lactogen receptor and displace 80–95% of the tracer at high concentrations (Fig. 2D), but fail to bind at significant levels to the somatogen receptor (Fig. 2C). Most notably in the lactogen system, the position and slope of the hGH-rP3 curve can be superimposed over that of native rPRL (Fig. 2, D and B, respectively). The lactogen receptor binding curves of hGH-rP4 and hGH-rG3 are both displaced further to the right than hGH-rP3 but maintain clearly detectable receptor binding activity.

DISCUSSION

The data presented in this report demonstrate that each of the chimeric genes expresses an appropriately sized, stable, secreted protein that binds to at least one of the two receptor model systems and is recognized by the polyclonal hGH antibody. This suggests that the exchange of corresponding exons within the GH-PRL family does not introduce major disruptions in protein structure. Furthermore, all three chimeric hormones selectively lose somatogen receptor binding activity (*i.e.* concentrations of chimera at least 3 orders of magnitude greater than hGH are required to displace 50% of ^{125}I-hGH) while retaining lactogen receptor binding activity. While the exact structural basis for this loss of activity remains to be defined, comparison of our results to previously published hGH mutagenesis experiments is informative. Extensive site-directed mutations of the hGH gene have defined three regions of hGH which contribute to the somatogen receptor binding domain, one each in exons 2, 3, and 5 (16). While the loss of somatogen receptor binding in our two exon 3 chimeras may reflect alterations in the exon 3 region previously described (amino acids 56–64), the loss of somatogen receptor binding activity in our exon 4 chimera (hGH-rP4) cannot be explained by the same formulation. The presence of important somatogen receptor binding de-

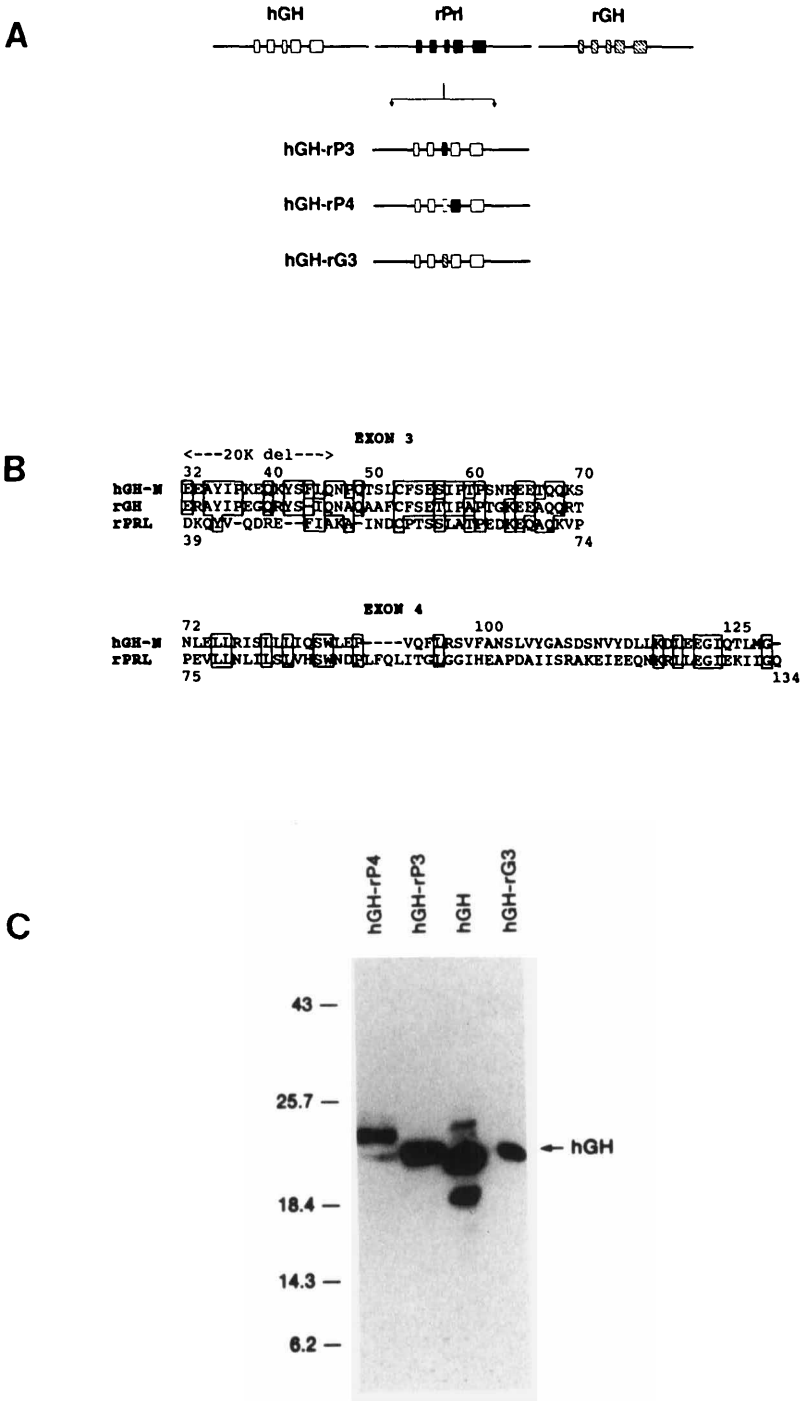

Fig. 1. Structure and Expression of hGH-Derivative Chimeric Proteins

A, Schematic representation of hGH-derivative exon exchange genes. Exons originating from the hGH gene are shown as *open boxes*, rPRL as *solid boxes*, and rGH as *cross-hatched boxes*. The genes are not drawn to scale. B, Alignment of the amino acid sequences encoded by exons 3 and 4 of the hGH, rGH, and rPRL genes. The amino acid sequences as derived from the gene sequences (1, 19–21) were aligned to maximize homology. Homologous residues are boxed. The 15 residues not present in the naturally occurring 20 kDa hGH variant are bracketed and labeled (20K del) (34, 35). C, Immunoblot analysis of the conditioned media from cell lines expressing hGH and hGH-derivative genes. Conditioned media from cell lines expressing hGH-rP4, hGH-rP3, hGH, and hGH-rG3 was resolved by polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose filters, and incubated with rabbit anti-hGH sera. Immune complexes were visualized by subsequent incubation with goat anti-rabbit immunoglobulin and ^{125}I-protein A. The lower band in the hGH-rP4 lane represents minor spillover from the adjacent hGH-rP3 lane and was not present in subsequent analytic gels of this sample. The lower band in the hGH lane represents the well described 20 kDa internal deletion isohormone (34) resulting from usage of an alternative splice-acceptor site in exon 3 (25, 35). The position of molecular weight markers is indicated to the left of the autoradiograph.

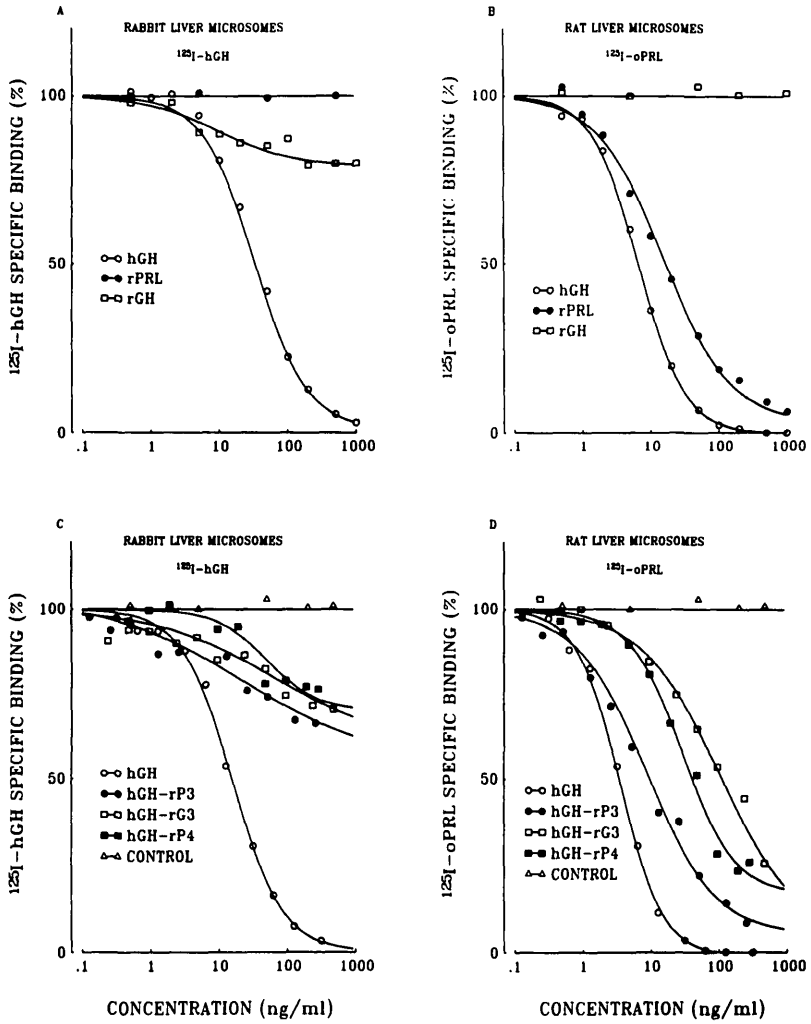

Fig. 2. Binding of hGH and hGH-Derivative Proteins to Somatogen and Lactogen Receptors

The ability of each of the recombinant proteins to bind to somatogen and lactogen receptors was measured by the ability of each conditioned media to block ^{125}I-hGH or ^{125}I-oPRL binding to crude membrane fractions prepared from adult rabbit livers and adult female rat livers, respectively. The control binding profiles of native hGH, rPRL, and rGH to these somatogen and lactogen receptor preparations are shown in A and B, respectively. Concentration of the recombinant hormone in each of the conditioned media was determined by ELISA as detailed in *Materials and Methods*.

terminants in exon 4 of hGH is, in fact, supported by studies which have demonstrated that a monoclonal antibody recognizing residues 98–128 blocks somatogen receptor binding (17) and by studies which demonstrate residual somatogen receptor binding activity in an exon 4 tryptic peptide (amino acids 97–108) (23). Our results and these reports suggest that regions encoded by exon 4 as well as 2, 3, and 5 of hGH contribute to somatogen receptor binding activity while exon 3 and possibly 4 may not be similarly critical to binding to the lactogen receptor.

Lactogen receptor binding is retained to some degree by all three chimeric hormones, while all three have essentially lost somatogen receptor binding. This suggests that the structural requirements for lactogen receptor binding may be less stringent than the requirements for somatogen binding. When hGH acquires PRL exon 3 (hGH-rP3), the slope of its binding curve shifts and becomes superimposable with that of native rPRL in the rat receptor system. PRL exon 3, therefore, may play an important modulating role in determining the precise fit between hormone and lactogen receptor. More remarkable, however, is the result with hGH-rG3. As demonstrated previously, rGH does not bind to the rat lactogen receptor. Its exon 3 encoded sequences can therefore be considered functionally null while at the same time contributing to proper preservation of the overall structure of the chimeric protein. Human GH-rG3 continues to bind to the lactogen receptor, albeit less well than hGH (its 50% displacement concentration is ~1.5 orders of magnitude greater than hGH). These data indicate that exon 3 sequences influence, but are not necessary for, lactogen binding specificity. Therefore, two or more regions of hGH encoded by exons 3 and 4 which are clearly required for somatogen receptor binding are distinct from those regions required by the less stringent lactogen receptor.

The results in this report suggest a greater complexity than previously appreciated in the distribution of the receptor binding domains of hGH. The somatogen receptor binding region of hGH may extend beyond the three discrete regions previously defined by mutational analysis and monoclonal antibody blocking studies (16). The specific loss of somatogen binding in hGH-rP4 suggests that determinants in exon 4 are also necessary for this activity. The somatogen receptor binding activity of hGH appears to be dependent on one or more determinant(s) which are distinct from the lactogen receptor binding region as three multi-residue substitutions in exons 3 and 4 (hGH-rP3, hGH-rP4, and hGH-rG3) all selectively destroy somatogen binding while maintaining lactogen binding to some degree. The structural requirements for lactogen receptor binding therefore appear to be distinct from those that determine somatogen receptor binding. Further structural modification of these hormones should result in clear disassociation of these two receptor binding domains.

MATERIALS AND METHODS

Recombinant DNA Manipulations

All recombinant manipulations were carried out using standard protocols (24). Restriction and modification enzymes were purchased from either Bethesda Research Laboratories (BRL, Gaithersburg, MD), or New England Biolabs (NEBL, Beverly MA) and were used as suggested by the supplier. *Xho*I and *Sal*I linkers were purchased from NEBL and were phosphorylated before use.

Construction of Exon Chimera Genes

The hGH-rP3 and hGH-rP4 genes were constructed as previously reported (18). In brief, exons 3 or 4 along with adjacent intron sequences were removed from the hGH gene and separately replaced with an *Xho*I linker. Exons 3 and 4 of the rPRL gene along with adjacent intron sequences were then isolated, ligated to *Xho*I linkers, and inserted into the corresponding hGH exon deletion genes at the XhoI site. To construct the hGH-rG3 gene, the third exon and adjacent intron sequences of the rGH gene were isolated on a *Bgl*II-*Pst*I restriction fragment (21), ligated to *Xho*I linkers and inserted at the *Xho*I site of the hGH gene containing an exon 3 deletion. Correct orientation of inserted exons was confirmed by sequencing across the ligation junctions. All splicing frames were unaltered by these exchanges.

Expression of Recombinant hGH and hGH-Derivative Proteins

Each of the hGH and hGH-derivative genes was transferred from a pBR322 subclone to the bovine papillomavirus (BPV)-mMT expression vector pBPV-MT-*Xho*I (pMT-X) (gift of D. Hamer, NIH, and described in Ref. 25). The GH gene was released from the pBR322 vector by digesting with *Bam*HI (cutting one base 3′ to the cap addition site of the hGH gene (1) and 375 base pairs 3′ to the *Eco*RI insertion site of pBR322), ligating *Sal*I linkers to blunted ends and ligating this 2.5 kilobase fragment to the *Xho*I site of the pMT-X vector [in the 5′ nontranslated region of the mMT gene (26)], thereby placing the hGH or hGH-derivative gene under the transcriptional control of the mMT promoter.

Establishment of Stable Cell Lines Expressing the hGH and hGH-Chimeric Genes

Each of the pMT-X-hGH recombinants was co-transfected into Cl27 mouse fibroblasts (American Type Culture Collection, Rockville, MD) with pRSVNeo at a 10:1 weight ratio by $Ca_3(P0_4)_2$ coprecipitation as previously described (2). Transfected cells were grown in the presence of 0.4 mg/ml G418 in minimal essential medium for 6 weeks, followed by isolation of individual foci by trypsinization in cloning rings. Ten or more individual foci from each transformation were expanded and assayed for secretion of hGH or hGH-derivative by enzyme-linked immunosorbent assay (ELISA) (see below) and the cell line with the highest expression was used to condition media.

Synthesis and Characterization of Recombinant Proteins

To condition media, each of the selected lines was grown to confluence and incubated overnight in serum-free media (HL-1 media, Ventrex Laboratories, Inc., Portland, ME). The size and integrity of each of the hormones used in the receptor binding assays was confirmed by immunoblotting. Fifty- to 250-μl aliquots of the media were resolved on a 15% polyacrylamide-sodium dodecyl sulfate (SDS) gel and were immunoblotted using a polyclonal rabbit anti-hGH primary antisera, goat anti-rabbit immunoglobulin as the secondary antibody, and ^{125}I-protein A for detection by autoradiography. Media were analyzed from cell lines expressing hGH-rG3 (50 μl), hGH (100 μl), hGH-rP3 (250 μl), or hGH-rP4 (250 μl) (Fig. 1C). For amino acid sequencing, 0.2 ml hGH and hGH-rP3 were electrophoresed through a preparative 12% polyacrylamide SDS gel, visualized by Coomassie blue staining, and the GH bands were excised from the gel and harvested by electroelution (27). Sequencing was carried out by W. Lane at the Microchemistry Facility, Harvard University (Cambridge MA).

The concentration of each of the GH-related proteins in the media was quantified by ELISA (28) in comparison to a standard curve generated from pituitary hGH (National Hormone Pituitary Agency; NIH hGH AFP 5180A, 2.2 IU/mg). A rabbit anti-hGH antiserum was used as primary antibody and peroxidase-conjugated goat anti-rabbit immunoglobulin (Zymed Laboratories., Inc., San Francisco, CA) as secondary antibody. The media concentrations were: hGH, 3.60 μg/ml; hGH-rP3, 1.34 μg/ml; hGH-rP4, 0.47 μg/ml; and hGH-rG3, 2.21 μg/ml. The sensitivities of immunological detection of each of the three chimeric proteins are equivalent and are approximately half that of hGH when concentrations are compared by silver-stained polyacrylamide gel electrophoresis of the conditioned media (data not shown). The background immunoreactivity in conditioned media from the control (vector alone) cell line was less than 1% of total signal. The ELISA values were used for the abscissa in Fig. 2.

Hormone Receptor Preparations

Crude membrane fractions (microsomes) were prepared from adult rabbit livers (5) and adult female rat livers (29). The rabbits were treated sc with four injections of bromocriptine (2 mg), starting 36 h before killing, to decrease endogenous PRL levels, and increase the concentration of unoccupied receptors. The rats were injected with 1 mg estradiol valerate sc 1 week before killing to increase the concentration of hepatic membrane receptors. As with the rabbits, they were also injected with 2 mg bromocriptine at 24 h, 12 h, and 30 min before killing. Microsomes were prepared by methods previously described (29, 30).

RRA

Pituitary hGH and oPRL were iodinated by the chloramine-T method (31) modified to use low concentrations of chloramine-T (32). Monomeric iodinated hormones, purified on Sephadex

G-75 columns had specific activities of 75–95 μCi/μg. Binding to the somatogen receptors on rabbit liver membranes was measured as competition with a [^{125}I]hGH tracer and binding to the lactogen receptors on rat liver was measured in competition with a [^{125}I]oPRL tracer. The conditioned media were diluted in assay buffer (25 mM Tris-HC1, pH 7.4, containing 10 mM $MgC1_2$ and 0.1% BSA) to obtain final concentrations of the hGH and hGH-derivative proteins in the ranges of 1–1000 ng/ml. For each assay point, 100 μl recombinant protein or standard hGH or oPRL at the appropriate dilution were incubated with membranes in the presence of 22,000–30,000 cpm [^{125}I]ligand for 17 h at 23 C. Control medium conditioned by cells stably transfected with the expression vector alone (cell line CM2–2) was diluted and assayed in parallel with the hGH-N conditioned media. All experiments were performed in duplicate at least twice. Bound and free hormone were separated by centrifugation at 3,000 rpm after dilution with 3 ml assay buffer. Specific binding was calculated as the difference between the radioactivity bound in the absence and presence of an excess of unlabeled ligand (1 μg hGH or oPRL). Data are presented as the percent specific [^{125}I]ligand binding to membranes in the absence of competitor. Competition curves were calculated on an IBM PC using the LIGAND program [LIGAND Program, Elsevier Biosoft, Cambridge England (33)].

Experimental Animals

All animal studies were conducted in accord with the principles and procedures as outlined in the NIH Guide for the Care and Use of Laboratory Animals.

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