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15. Slowly exchanging NH protons were identified by recording a series $^1H$-$^{15}N$ Overbodenhausen correlation spectra [G. Bodenhausen and D. J. Ruben, *Chem. Phys. Lett.* **69**, 185 (1980); A. Bax, M. Ikura, L. E. Kay, D. A. Torchia, R. Tschudin, *J. Magn. Reson.* **86**, 304 (1990)] over a period of ~24 hours starting within 5 min of dissolving an unexchanged sample of lyophilized protein in $D_2O$.
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# Rational Design of Potent Antagonists to the Human Growth Hormone Receptor

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**A hybrid receptor was constructed that contained the extracellular binding domain of the human growth hormone (hGH) receptor linked to the transmembrane and intracellular domains of the murine granulocyte colony-stimulating factor receptor. Addition of hGH to a myeloid leukemia cell line (FDC-P1) that expressed the hybrid receptor caused proliferation of these cells. The mechanism for signal transduction of the hybrid receptor required dimerization because monoclonal antibodies to the hGH receptor were agonists whereas their monovalent fragments were not. Receptor dimerization occurs sequentially—a receptor binds to site 1 on hGH, and then a second receptor molecule binds to site 2 on hGH. On the basis of this sequential mechanism, which may occur in many other cytokine receptors, inactive hGH analogs were designed that were potent antagonists to hGH-induced cell proliferation. Such antagonists could be useful for treating clinical conditions of hGH excess, such as acromegaly.**

Knowledge of the molecular basis for hormone action is key to the rational design of hormone agonists and antagonists. High-resolution mutational analysis (*1*, *2*) and x-ray crystallographic studies (*3*) have defined two sites on hGH for binding two molecules of the extracellular domain of its receptor (hGHbp) (*4*). Dimerization of the hGHbp occurs sequentially, such that a hGHbp molecule binds to site 1 and then a second hGHbp molecule binds to both site 2 on hGH and a site on the first hGHbp (Fig. 1). A thorough examination of the biological importance of this model has been precluded because of the lack of an adequate cellular signaling assay for hGH. Here, we constructed a sensitive, cell-based assay for hGH, investigated the mechanism for signal transduction, and applied the assay for the design of antagonists to the hGH receptor.

The hGH receptor belongs to a large family of receptors of hematopoietic origin (*5*) that includes the interleukin-3 (IL-3) and granulocyte colony-stimulating factor (G-CSF) receptors. An IL-3–dependent myeloid leukemia cell line (FDC-P1) transfected with the full-length murine G-CSF (mG-CSF) receptor is stimulated to proliferate by G-CSF without IL-3 (*6*). We constructed a hybrid receptor that contained the hGHbp linked to a portion of the mG-CSF receptor containing the three extracellular fibronectin repeats and the transmembrane and intracellular domains (*7*). The fibronectin domains do not participate in the binding of G-CSF but are required for efficient expression of the mG-CSF receptor (*6*).

Competitive displacement of $^{125}I$-labeled hGH from hybrid receptors on whole cells was used to establish the affinity for hGH and the approximate number of receptors per cell (*8*). In several independent

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binding experiments, the apparent dissociation constant ($K_d$) value for hGH was 0.1 ± 0.03 nM, and there were 1000 ± 300 receptors per cell. This affinity is about three to four times stronger than that for hGH binding to the soluble hGHbp and may reflect a high local concentration of receptors on cells (an avidity effect). Nontransfected cells lacked specific binding sites for hGH (9). At low concentrations, hGH induces cell proliferation with a median effective concentration ($EC_{50}$) of ~20 pM (Fig. 2A), a value somewhat lower than the apparent $K_d$ for binding to whole cells (~100 pM). This may indicate that signaling for maximal cell proliferation requires less than total receptor occupancy.

Each hGH molecule is bivalent because it contains two separate sites for binding the hGHbp (Fig. 1). In contrast, the hGHbp is effectively univalent because each site uses virtually the same determinants to bind to either site 1 or site 2 on hGH (3). Excess hGH will dissociate the hGH·$(hGHbp)_2$ complex to form a hGH·hGHbp complex in which hGH is bound exclusively at site 1 to the hGHbp (1). Thus, excess hGH should antagonize signaling by preventing dimerization (Fig. 1). Indeed, at very high hGH concentrations the proliferation activity is lost [concentration required to inhibit proliferation by 50% ($IC_{50}$) ≅ 2 μM]. Cell proliferation induced by IL-3 was not altered in the presence of high concentrations of hGH (8 μM); thus, 8 μM hGH is not toxic to cells (9). This effect appears not to involve cross-linking of receptors between cells or other cell-to-cell interactions because the effects of hGH were not influenced by cell density. Furthermore, the assay is specific because FDC-P1 cells that contain the full-length mG-CSF receptor do not respond to hGH and cells that contain the hybrid receptor do not respond to G-CSF (10).

To further investigate the requirement

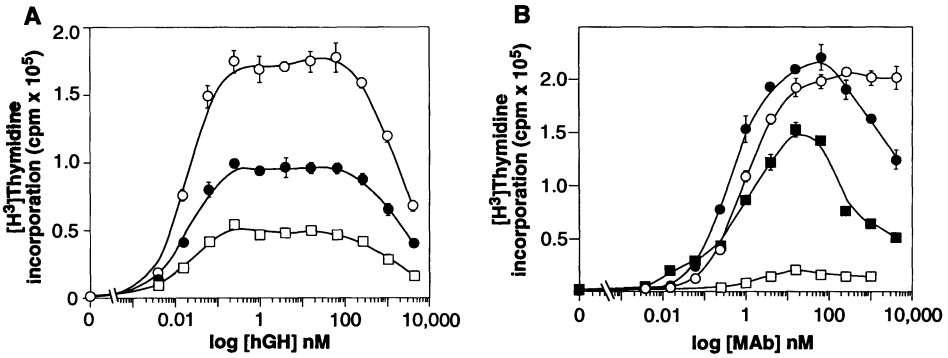


**Fig. 2.** Proliferation of FDC-P1 cells containing the hGH–mG-CSF hybrid receptor induced by hGH (7) (**A**) or induced by MAbs to the hGH receptor (**B**) (17). In (A), cells were grown in RPMI 1640 media supplemented with IL-3 (10 U/ml), 10 μM β-mercaptoethanol, and 10% FBS at 37°C and 5% $CO_2$ (6). Cells were washed with the same medium without IL-3. Cells were added to 96-well plates at a density of 4 × $10^5$ cells per milliliter (○), 2 × $10^5$ cells per milliliter (●), and 1 × $10^5$ cells per milliliter (□) in 100 μl. Cells were then treated with various concentrations of hGH for 18 hours. To measure DNA synthesis, we added $^3$H-labeled thymidine (1 μCi per well) to each well. After 4 hours, cells were collected and washed on glass filters. Scintillation cocktail (2 ml) was added, and radioactivity was counted with a Beckman LS1701 scintillation counter. In (B), cells were cultured as in (A) and plated at a density of 4 × $10^5$ cells per milliliter in medium containing various concentrations of anti-hGH receptor MAb 263 (●), MAb 13E1 (○), MAb 3D9 (■), or MAb 5 (□). After 18 hours at 37°C, cells were washed, and the amount of [$^3$H]DNA synthesized was determined by scintillation counting as in (A). Each data point represents the mean of triplicate determinations, and error bars indicate the SD.

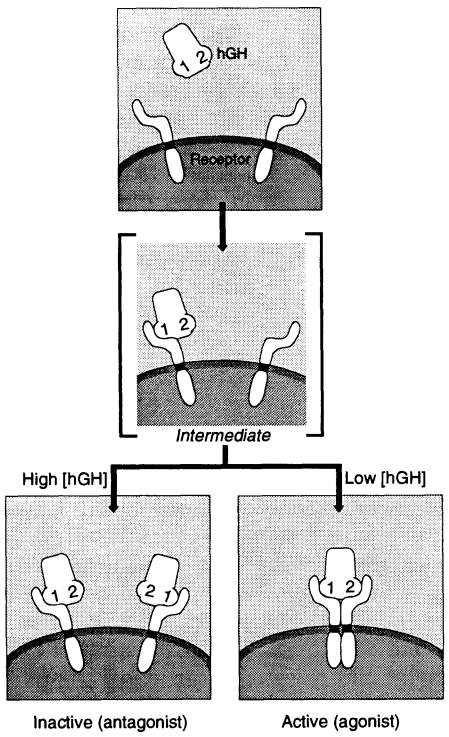


**Fig. 1.** Sequential dimerization model for activation of the hGH–mG-CSF hybrid receptor. At low concentrations, hGH binds first at site 1 and subsequently at site 2 (as indicated) to produce an active hGH·$(hGHbp)_2$ complex. At high concentrations, hGH saturates the receptor through site 1 interactions and acts as an antagonist. We show the receptors dissociated initially because, in the absence of hGH, the hGHbp does not self-dimerize as shown by ultracentrifugation for concentrations <0.1 mM. Nonetheless, it is possible that some full-length receptors are loosely pre-dimerized and become activated upon sequential binding of hGH.

**Table 1.** Summary of dose-response data for a variety of anti-hGH receptor MAbs, FAbs (17), and hGH mutants (12) for stimulating proliferation of FDC-P1 cells containing the hGH–mG-CSF hybrid receptor. "None" indicates that no effect was observed; ND, not determined. $K_d$ values for MAbs binding to the hGHbp were taken from (14). The $K_d$ values for hGH and variants were measured with a competitive displacement assay in which $^{125}$I-labeled hormone bound to hGHbp was precipitated with MAb 5 (2, 13). This gives the affinity for the monomeric hGH·hGHbp complex. Values for $EC_{50}$ were taken from titration curves shown in Fig. 2, A and B, and Fig. 4 and represent the half-maximal concentration for stimulation of cell proliferation. Data are the mean of triplicate assays, and the SDs were within 15% of mean. Values shown with > indicate that maximal stimulation or inhibition of proliferation was not detected at the concentrations tested. For these cases, we report only estimates of the $EC_{50}$. $IC_{50}$ refers to the concentration leading to 50% inhibition of maximal cell proliferation.

| Protein | $K_d$ (nM) | $EC_{50}$ | $IC_{50}$ (self-antagonism) |
|---|---|---|---|
| MAb 263 | 0.6 | 0.3 nM | ~3 μM |
| MAb 13E1 | 3.2 | 0.8 nM | >10 μM |
| MAb 3D9 | 2.2 | 0.8 nM | 0.2 μM |
| MAb 5 | 0.7 | ~2.5 nM | >1 μM |
| FAb 263 | ND | >1.5 μM | ND |
| FAb 13E1 | ND | >3 μM | ND |
| FAb 3D9 | ND | >0.1 μM | ND |
| FAb 5 | ND | >1 μM | ND |
| hGH | 0.3 | 20 pM | 2 μM |
| K172A/F176A | 200 | 25 nM | None |
| G120R | 0.3 | None | None |
| H21A/R64K/E174A | 0.01 | 20 pM | 60 nM |
| H21A/R64K/E174A/G120R | 0.01 | None | None |

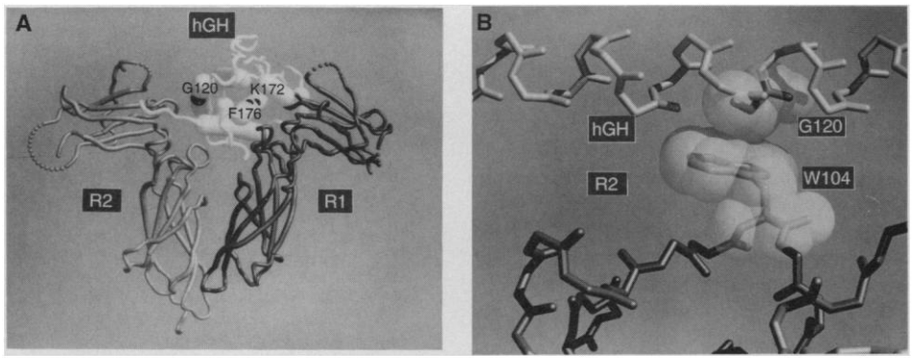


**Fig. 3.** Molecular models based on x-ray crystallography of hGH bound to the hGHbp (*3*). (**A**) A ribbon diagram of hGH (white) bound to two molecules of the extracellular domain of the hGH receptor (hGHbp; gray and black). The α carbon positions of mutant residues in hGH are shown by black dots. K172 and F176 are located in site 1 (interface with black hGHbp) and G120 is located in site 2 (interface with gray hGHbp). Gray dots indicate structures in the hGHbp that are not well defined by the electron density. The model is based on a 2.7 Å resolution x-ray structure of the complex (*3*). (**B**) A close-up showing that G120 located on helix 3 of hGH makes van der Waals contact with W104 from the hGHbp bound to site 2. R1 and R2, receptor 1 and receptor 2, respectively.

**Table 2.** Summary of antagonist effects of FAbs and hGH analogs that block hGH-induced cell proliferation of FDC-P1 cells containing the hybrid hGH–mG-CSF receptor. Cells were incubated with 1 nM hGH and various concentrations of FAb (*17*) or hGH analog (*12*). The $IC_{50}$ is the concentration required to block 50% of the cell proliferation activity of hGH. "None" indicates that no inhibition was observed at concentrations of FAb or hGH analog of up to 10 μM.

| Protein | $IC_{50}$ |
|---|---|
| FAb 263 | None |
| FAb 13E1 | 0.8 μM |
| FAb 5 | 0.2 μM |
| FAb 3D9 | None |
| hGH | 2 μM |
| K172A/F176A | None |
| G120R | 20 nM |
| H21A/R64K/E174A | 60 nM |
| H21A/R64K/E174A/G120R | 2 nM |

for dimerization of the hGHbp to signal in the hybrid receptor cell proliferation assay, we used bivalent monoclonal antibodies (MAbs) and univalent fragments derived from them (FAbs) that recognized the hGHbp. At low concentrations, three of four different MAbs to the receptor were as potent as hGH in inducing cell proliferation (Fig. 2B and Table 1). The $EC_{50}$ value for each MAb (0.3 to 1 nM) was usually somewhat less than the $K_d$ value determined by enzyme-linked immunosorbent assay (Table 1). As with hGH, this may reflect avidity effects on whole cells or that maximal signaling is achieved at less than 100% receptor occupancy, or both. At much higher concentrations (0.2 to ~3 μM), two of these MAbs were less effective at stimulating proliferation, presumably because excess MAb blocks receptor cross-linking by binding monovalently to hGHbp. Corresponding monovalent FAb fragments had little or no effect on cell proliferation (Table 1), which indicates further that bivalency is required for signaling activity.

The differences in stimulation of cell proliferation at low concentrations and inhibition at high concentrations for these MAbs (Fig. 2B) can be explained by the different ways they bind to the hGHbp. MAb 5 prevents binding of a second hGHbp to the hGH·hGHbp complex (*1*), possibly by binding to the region where both receptors contact each other (Fig. 1). The fact that MAb 5 is the least efficient at stimulating proliferation may indicate that the receptors need to approach each other closely for optimal signaling. MAb 13E1 did not inhibit proliferation at the concentrations tested. This MAb blocks hGH binding (*11*) and probably binds like hGH to form very stable receptor dimers. In contrast, MAbs 263 and 3D9 bind at sites away from the hormone-receptor interfaces (*11*) and show similar agonistic and antagonistic effects on proliferation. Maximal stimulation of proliferation by hGH occurred over a wider range of concentrations than did maximal stimulation by MAbs 263 and 3D9, perhaps because with hGH bound, the dimers have the optimal receptor-to-receptor contacts. The fact that MAbs 263 and 3D9 are agonists suggests that the structural constraints for formation of active dimers are rather loose.

FAb fragments derived from MAb 13E1 or MAb 5 antagonized hGH-induced cell proliferation, whereas those derived from MAbs 263 and 3D9 did not (Table 2). These studies are consistent with the fact that the binding of MAb 13E1 or MAb 5 to their epitopes blocks hormone-to-receptor or receptor-to-receptor interfaces, respectively.

To determine the structural requirements for dimerization of hGH (Fig. 1), we examined mutants of hGH that were designed to reduce binding of the receptor to site 1 or site 2 (Fig. 3). The mutant K172A/F176A (*12*), which preserves site 2 determinants but alters important side chains in site 1, promoted cell proliferation, but the $EC_{50}$ was shifted to a concentration about $10^3$ times higher than that of wild-type hGH (Fig. 4 and Table 1). This is consistent with the 560-fold reduction in the affinity for site 1 binding of the K172A/F176A mutant as compared to that of the wild-type hGH when measured in vitro (*13*). No inhibition of proliferation with K172A/F176A was observed at the concentrations tested.

On the basis of the x-ray structure of the hGH·$(hGHbp)_2$ complex (*3*), we designed

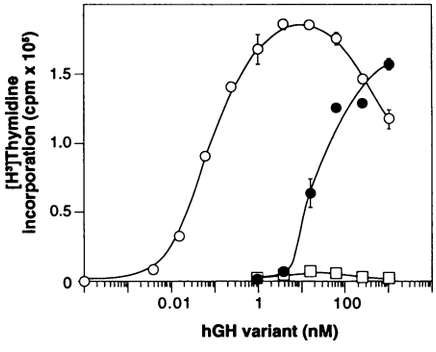


**Fig. 4.** Proliferation of FDC-P1 cells containing the hGH–mG-CSF hybrid receptor, caused by increasing concentrations of wild-type hGH (○), the site 1 hGH variant K172A/F176A (●), and the site 2 hGH variant G120R (□). Cells were cultured, treated, and assayed as described in Fig. 2A, except that cells were treated for 18 hours with [³H]thymidine. The hGH mutants were prepared and purified as described (*2, 12*).

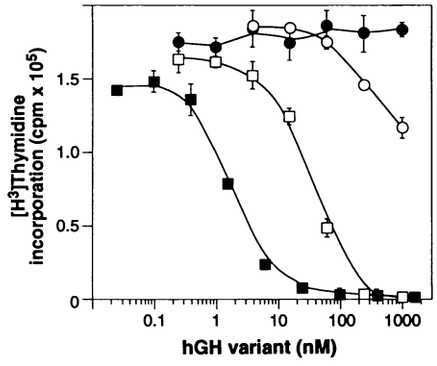


**Fig. 5.** Antagonism of hGH-induced cell proliferation by hGH variants. Cells were prepared as in Fig. 2A and incubated with 1 nM hGH and various concentrations of the site 1 mutant K172A/F16A (●), the site 2 mutant G120R (□), the combined enhanced site 1 and site 2 mutant (H21A/R64K/E174A/G120R) (■), and wild-type hGH (○).

a mutant G120R, which retains a functional site 1 but on which site 2 is sterically blocked (Fig. 3B). This variant did not affect cell proliferation at the concentrations tested (Fig. 4). Thus, binding to either site 1 or site 2 is necessary but not sufficient for promoting cell proliferation.

If the sequential signaling mechanism (Fig. 1) is correct, mutants blocked in site 2 binding (but not in site 1 binding) should antagonize hGH-induced cell proliferation. To test this, we cultured cells with enough hGH (1 nM) to support 90% of maximal cell proliferation and added increasing concentrations of wild-type hGH or the mutants in site 1 (K172A/F176A) or site 2 (G120R). As expected, the site 2 mutant antagonized hGH whereas the site 1 mutant was ineffective (Fig. 5). In fact, the site 2 mutant was nearly 100 times more potent as an antagonist ($IC_{50}$ = 20 nM) (Table 2) than wild-type hGH ($IC_{50}$ = 2 μM). For hGH to be antagonistic, free hormone must react with free receptors before the hGH-bound intermediate does so. This only occurs at high concentrations of hGH. In contrast, once G120R is bound, it cannot dimerize and agonize the receptor. Thus, G120R as an antagonist does not need to compete against G120R as an agonist.

Although G120R is a much more potent antagonist than hGH, 20 nM G120R was required to inhibit by 50% the proliferative effect of 1 nM hGH (Table 2). This may reflect the fact that hGH is bound through interaction of sites 1 and 2 with two receptors more tightly than G120R is bound in the complex with a single receptor through site 1 alone. Furthermore, maximal signaling by hGH may not require 100% receptor occupancy. In either case, improving the affinity of site 1 for hGHbp in the G120R mutant should make it a more potent antagonist.

Single-site hGH variants have been produced (*2*, *14*) that bind more tightly to the hGHbp at site 1. A variant that contains all three of these mutations (H21A/R64K/E174A) bound 30 times more tightly than wild-type hGH to the hGHbp (Table 1). This variant had an $IC_{50}$ for inhibiting proliferation that was about 30 times lower than that of hGH. This is consistent with the notion that the inhibitory effect results from competition for binding to hGHbp between site 2 on the bound hormone-receptor intermediate and the free site 1 on the soluble hormone. The fact that improvement in site 1 binding affinity did not improve the efficacy of the hormone as an agonist may be understood upon future analysis of the on and off rates.

We further mutated this variant by changing $Gly^{120}$ to Arg. The mutant with all four modifications was ten times more potent than G120R as an hGH antagonist (Fig. 5 and Table 2). This is further evidence for the importance of site 1 binding properties for antagonism.

Our data suggest that the inhibition of proliferation caused by hGH, MAbs, and their derivatives is the result of blocking receptor dimerization rather than causing down-regulation of receptors. First, cells propagated with IL-3 instead of hGH do not show a greater hGH response or hGH receptor number (*9*). Second, receptor down-regulation is usually correlated to receptor activation. The ratio of $EC_{50}$ to $IC_{50}$ for each of the MAbs and hGH varies widely, which shows that receptor activation can be readily uncoupled from inhibition by the alteration of binding properties. Finally, the G120R mutant is inactive as an agonist, although it is a more potent antagonist than hGH (Fig. 5), and pretreatment of cells with G120R does not enhance its antagonistic effect (*9*). Thus, the antagonistic effect of G120R is not consistent with receptor down-regulation. It is possible that the inhibitory effects observed for other hormones at high concentrations may occur because receptor dimerization is blocked by self-competition.

Our studies indicate that sequential dimerization is crucial for hybrid-receptor activation. Knowledge of this mechanism and the structural (*3*) and functional (*1*, *2*) properties of the binding interfaces allowed us to design potent antagonists to the hGH receptors, which may be useful in the clinical treatment of hGH excess acromegaly (*15*). In fact, a transgenic strain of mice that expresses large amounts of bovine GH altered in site 2 produces dwarf mice (*16*). This mechanism-based strategy for design of potent antagonists for hGH may be applicable to other hormones such as prolactin, placental lactogen, IL-2, IL-3, IL-6, G-CSF, granulocyte-macrophage–CSF, erythropoietin, and related hematopoietines and cytokines (*5*) if sequential binding of two receptors to a single hormone molecule is required for their signaling.

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7. A hybrid receptor was constructed from cDNA that contained exons 1 through 7 of the hGH receptor (which encode the secretory signal and the extracellular hGH binding domains) linked to exons 9 through 17 of the mG-CSF receptor (which encode the three fibronectin domains and the entire transmembrane and intracellular domains). The protein encoded by the hybrid cDNA contains the amino acids −18 to 243 of the hGH receptor and 309 to 812 of the mG-CSF receptor. In this construction, $Cys^{241}$ was replaced by Arg, which has no effect on binding to hGH [G. Fuh *et al.*, *J. Biol. Chem.* **265**, 3111 (1990)]. Sequences derived from the hGH receptor [D. W. Leung *et al.*, *Nature* **330**, 537 (1987)] and from the mG-CSF receptor [R. Fukunaga *et al.*, *Cell* **61**, 341 (1990)] were cloned by means of the polymerase chain reaction [R. Higuchi, in *PCR Protocols: A Guide to Methods and Applications*, M. Innis *et al.*, Eds. (Academic Press, New York, 1989), pp. 177–183] into the vector pER-BOS (*6*). The hybrid cDNA was introduced into mouse FDC-P1 cells as described (*6*). Stable transformants that expressed the hybrid protein were identified by their ability to bind $^{125}$I-hGH (*8*).
8. Cells grown with IL-3 were washed before the assay with phosphate-buffered saline (PBS) that contained 10% fetal bovine serum. Cells ($1.2 \times 10^6$ per milliliter) were incubated with serial dilutions of hGH in the presence of $^{125}$I-labeled hGH variant Y103A (20 pM) for 18 hours at 4°C. The Y103A variant was used to prevent iodination of Y103, which inhibits the binding of the receptor to site 2 (*9*). Cells were washed with PBS twice to remove the excess hormone, and the bound radioactivity was counted.
9. G. Fuh and J. A. Wells, unpublished results.
10. E. Ishizaka-Ikeda, R. Fukunaga, S. Nagata, unpublished results.
11. S. H. Bass, M. G. Mulkerrin, J. A. Wells, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4498 (1991).
12. Mutants of hGH were prepared by site-directed mutagenesis [T. A. Kunkel, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987)] and purified as described (*2*, *13*). Mutants are designated by the wild-type residue followed by its position and the mutant residue in the single-letter code A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Try; and Y, Tyr. Thus, G120R indicates that $Gly^{120}$ was converted to Arg. Multiple mutants are represented by a series of single mutants separated by slashes.
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16. W. Y. Chen, D. C. Wright, B. V. Mehta, T. E. Wagner, J. J. Kopchick, *Mol. Endocrinol.* **5**, 1845 (1991).
17. The MAbs 5 and 263 were from Agen (Parsippany, NJ) and have been described [R. Barnard, P. G. Bundesen, D. B. Rylatt, M. J. Waters, *Endocrinology* **115**, 1805 (1984); *Biochem. J.* **231**, 459 (1985)]. The MAbs 13E1 and 3D9 were from the Genentech hybridoma group, and their properties have been described (*1*, *11*). Briefly, MAbs were purified from mouse ascites fluid by binding to Protein A–Sepharose (Pharmacia) and eluted with 0.1 M acetate (pH 3.0). FAb fragments were prepared by treating MAbs with dithiothreitol-activated papain (1 part to 50 parts MAb, by weight) in PBS with 10 mM cysteine for 1 hour. Digestions were stopped by adding 0.1 M iodoacetamide. The Fc and residual MAb were removed by adsorption onto Protein A–Sepharose twice, and the FAb fragments were further purified by gel filtration on Superose 12 (Pharmacia).


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