Activation of Chimeric and Full-length Growth Hormone Receptors by Growth Hormone Receptor Monoclonal Antibodies

A SPECIFIC CONFORMATIONAL CHANGE MAY BE REQUIRED FOR FULL-LENGTH RECEPTOR SIGNALING*

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Signal transduction by the growth hormone receptor (GHR) occurs through growth hormone (GH)-induced dimerization of two GHRs to form a trimeric complex. It is thought that dimerization alone is sufficient for signaling, since monoclonal antibodies (mAbs) against the extracellular domain of the GHR elicit proliferation of FDC-P1 cells transfected with a chimeric receptor comprising the extracellular domain of the GHR and the fibronectin and cytoplasmic domains of the murine granulocyte colony-stimulating factor receptor. We have screened 14 GHR mAbs for proliferative activity against characterized FDC-P1 and BaF-B03 cell lines stably expressing the full-length human, rabbit, or rat GHR, or the chimeric human GHR/granulocyte colony-stimulating factor receptor, and for transactivation of the c-*fos* promoter and STAT activation. With the chimeric receptor, eight mAbs were able to elicit proliferation, although there was no correlation between inhibition of hormone binding and agonist activity. In contrast, no mAbs were able to act as agonists with the full-length GHR FDC-P1 cell lines, although nine competed with GH for binding. A weak proliferative response was observed in the BaF-B03 cell lines with two of the mAbs (263 and 1C9), and the addition of anti-mouse F(ab)₂ resulted in increased signaling in the hGHR BaF-B03 cell line to a plateau of 28 ± 4% of the GH maximum for mAb 263. These data could indicate considerable stringency in the ability of mAbs to correctly dimerize the full-length GHR. However, the ability of mAb 263 to stimulate a mutant hGHR altered in the F′–G′ loop of domain 2 was nearly abolished, concurrent with an increased affinity of this mAb for the receptor. Since the F′–G′ loop undergoes a conformational change on GH binding and is necessary for full proliferative signaling, we propose that in addition to promoting receptor dimerization, mAb 263 may induce specific changes in receptor conformation similar to GH, which are required for the biological response.

Growth hormone (GH)[1] regulates a wide range of processes including somatic growth, metabolism, and synthesis of specific proteins (1). It does this by triggering multiple second messenger pathways in response to ligand binding to the GHR. The first signaling step after ligand binding is thought to be hormone-induced dimerization of identical receptor subunits. It has been shown that formation of the trimeric complex (GH·(GHR)₂) is a sequential process involving the binding of the hormone, first to receptor 1 and then to receptor 2, on opposite sides of the four-α-helical bundle hormone. Receptor binding sites on the hormone are referred to as sites 1 and 2, respectively (2, 3). Evidence supporting a role for hormone-induced dimerization in signaling was first provided by Fuh *et al.* (4) using a cell line expressing the extracellular binding domain of the GHR fused to the extracellular fibronectin and cytoplasmic domains of the murine granulocyte colony-stimulating factor (mG-CSF) receptor. These workers reported that a hGH analogue mutated in binding site 2 was unable to transmit a proliferative signal because it was unable to induce dimerization. Similarly, our mutagenic analysis of the dimerization domain that links dimerized receptors in the trimeric complex, demonstrates that receptor dimerization is necessary for signal transduction (5). The question arises, however, as to whether dimerization alone is sufficient, or whether there are additional hormone-induced conformational changes in the receptor required for signaling. The study of Fuh *et al.* (4) showed that three mAbs raised against the GHR could elicit cell proliferation in FDC-P1 myeloid cells containing the chimeric hGHR/G-CSF receptor, presumably by cross-linking two receptors in the same manner as GH (2, 3). Accordingly, monovalent Fab fragments of these mAbs were incapable of eliciting a biological response. This supports the notion that receptors are easily activated by cross-linking and that the constraints for signaling are minimal.

Because Fuh *et al.* (4) utilized the chimeric hGHR/G-CSF receptor in their study, it is not clear whether these mAbs can

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[1] The abbreviations used are: GH, growth hormone; Ab, antibody; mAb, monoclonal antibody; bGH, bovine growth hormone; G-CSF, granulocyte colony-stimulating factor; mG-CSF, murine G-CSF; GHBP, growth hormone-binding protein; GHR, growth hormone receptor; hGH, human growth hormone; hGHR, human growth hormone receptor; IL-3, interleukin 3; rbGHR, rabbit growth hormone receptor; FCS, fetal calf serum; PBS, phosphate-buffered saline; APRE, acute phase response element; EMSA, electrophoretic mobility shift assay.

activate the full-length GHR or whether the ability of these cells to respond to the GHR mAbs is in fact characteristic of G-CSF signaling. This is relevant, because we have previously been unable to find agonist activity of one of the mAbs used in this study (mAb 263) in a system measuring the insulin-like actions of GH (6). We have also found that a region of the extracellular domain of the GHR that undergoes a conformational change upon hormone binding (as visualized in the crystal structure) is an important determinant for proliferative signaling in FDC-P1 cells (7). This region, the F′–G′ loop in domain 2 of the GHR, is displaced in site 1 of the GHR but not in site 2, presumably as a result of the close interaction with the hormone at site 1 but not at site 2 (8). Modification of this loop has little affect on the ability of GH to form the trimeric GH·(GHR)$_2$ complex, but the ability of this receptor to activate a maximum proliferative response in FDC-P1 cells expressing this receptor is markedly attenuated (7). This observation suggests that activation of the GHR requires more than simple dimerization and that specific conformational changes in the GHR take place that are necessary for effective signaling. To further test this hypothesis, we have examined here the ability of 14 mAbs directed to the extracellular domain of the GHR to induce a biological response in the same cell line used by Fuh *et al.* (4) (hGHR/mG-CSF receptor) and in FDC-P1 and BaF-B03 cells expressing the full-length human, rabbit, and rat GHRs. We conclude that it is possible for mAbs raised against the extracellular domain of the GHR to initiate a biological response through dimerization of two GHRs. However, since only 2 of 14 mAbs were able to signal through the full-length receptor, and the response was not of the same magnitude as GH alone, we propose that a full biological response through the GHR is dependent on specific hormone-induced conformational change(s).

MATERIALS AND METHODS

Establishment and Characterization of Cell Lines Expressing the Human, Rabbit, and Rat Growth Hormone Receptors and the Chimeric hGHR/mG-CSF Receptor—FDC-P1 cells containing the chimeric hGHR/mG-CSF receptor (HC-2 cells) as used in Refs. 4 and 9 were a gift from Prof. S. Nagata (Osaka Biosciences Institute, Japan). The FDC-P1 cells expressing the rabbit GHR (FDC-P1-RGHR3B cells) are described in Ref. 8. A clone expressing the full-length human GHR (FDC-P1-HGHRD11 cells) was produced and characterized in the same manner described in Ref. 10, using the hGHR cDNA in the PECE expression vector (generously provided by Michel Goossens, INSERM 94010, Creteil, France). A clone expressing the rat GH receptor (11) was likewise generated in FDC-P1 cells. BaF-B03 cells (a gift from Dr. Tom Gonda (Hanson Institute, Adelaide, Australia)), were likewise co-transfected with p-Neo expression vector and the rGHR-pECE, hGHR-pECE, or Q2166 R2176 del218 hGHR-pECE (loop delete (7), constructed by Altered Sites, Promega) construct by electroporation as described for FDC-P1 cells. After G418 selection (0.3 mg/ml for FDC-P1, 1.2 mg/ml for BaF-B03 cells) and cloning by limiting dilution, binding assays utilizing ^{125}I-hGH were performed on individual clones to detect positive GHR-expressing lines. Further characterization was performed on each clone to determine receptor number and affinity as described for FDC-P1 cells above (10). All cell lines were maintained in phenol-free RPMI 1640 with 5% FCS, 1 μg/ml gentamicin at 37 °C, and 5% CO$_2$ with either recombinant hGH (Bresatec Ltd., Adelaide, Australia) at 40 ng/ml or recombinant IL-3 (a generous gift of Dr. Andrew Hapel at the John Curtin School of Medical Research, Canberra, Australia) at 100 units/ml.

Preparation of mAbs—mAbs 1, 5, 7, 43, 44, and 263 have been extensively characterized (12–14) and were supplied by Agen Ltd. (Acacia Ridge, Brisbane, Australia). mAb 1C9 was an anti-idiotype mAb, raised against a rabbit anti-hGH Fab (15), while all other anti-human receptor mAbs used in this study were raised against full-length recombinant nonglycosylated hGH receptor (residues 1–246) but known to cross-react with native hGH-binding protein (16). Anti-hGH mAbs 20 and 21 (17) were a generous gift of Dr. Jim Wells (Genentech, S. San Francisco, CA). All mAbs were Protein A-purified in the manner described in Ref. 18 and underwent extensive dialysis against PBS and

filter sterilization before use. Prior to cell proliferation and binding assays, samples of each mAb were run on a 12.5% SDS-polyacrylamide gel (19) under both reduced and nonreduced conditions to ensure their integrity.

Precipitation and Inhibition Assays with mAbs—As a means to determine that the mAbs used were able to recognize the GHR, precipitation assays were performed as described previously (12–14). Rabbit liver cytosol was prepared as described in Ref. 14, while recombinant human GHBP was a gift from Dr. Steve Spencer (Genentech).

Inhibition assays were performed with ^{125}I-bGH (for somatotrophic receptor) in the manner described in Ref. 12 with male rabbit liver microsomes prepared by the protocol described in Ref. 14. Inhibition assays with the chimeric receptor- and hGHR-expressing cells were performed with ^{125}I-hGH in the same manner, except physiological binding buffer supplemented with 2 mM MgCl$_2$ (8) was used. Cells were washed three times with an equal volume of PBS and resuspended in physiological buffer before 100-μl aliquots containing approximately 3×10^7 cells/ml were added to each assay tube. Assays were terminated by the addition of 2 ml of ice-cold physiological buffer (18) followed by centrifugation at $1600 \times g$, removal of the supernatant, and counting of the pellet in an LKB 1274 auto-γ-counter (LKB-Producer AB, Bromma, Sweden).

To determine if the epitope for mAb 263 was affected by the F′–G′ loop mutated hGHR, Scatchard analysis was performed with ^{125}I-mAb 263 as described above in the characterization of the GHR-expressing clones, with ^{125}I labeling of mAb 263 as described by Gobius *et al.* (18).

Cell Proliferation Assays—Cell proliferation was assessed using the MTT assay (20) as described in Ref. 10 except that a final concentration of FCS of 0.5% was used to decrease the background resulting from bovine GH in the FCS. The ability of the mAbs to stimulate cell proliferation was tested in the presence or absence of GH, as follows. To 50 μl of PBS-washed cells (8×10^5 cells/ml for FDC-P1 cells and 4×10^5 cells/ml for BaF-B03 cells) was added 50 μl of mAb followed 30 min later by 50 μl of media either devoid of or containing GH. A submaximal concentration of GH (25 ng/ml of porcine GH for rabbit GHR-expressing FDC-P1 cells, 2.5 ng/ml hGH for human GHR- and the chimeric receptor-expressing FDC-P1 cells, and 0.3 ng/ml of hGH for GHR-expressing BaF-B03 cells) was used in assays to determine the effects of mAbs on the GH response. This latter procedure was utilized in case of synergy with GH to increase the rate of GH·(GHR)$_2$ complex formation. For cell proliferation assays utilizing anti-mouse second Ab (anti-(H + L) affinity-purified; The Binding Site, Birmingham, UK), this was added 15 min after the addition of the primary mAb. After a 24-h incubation, 50 μl of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide at a concentration of 4 mg/ml was added to each well, and after a further incubation for 3 to 4 h in the dark, cells were lysed with 120 μl of isopropyl alcohol. The absorbance of the oxidized dye was measured on a model 450 microplate reader (Bio-Rad Laboratories Pty Ltd, North Ryde, Australia) at 595 nM.

Assays involving the anti-hGH mAbs 20 and 21 (17) were performed with BaF-B03 hGHRB2B2 cells at a density of 2×10^4 cells/well and hGH at a final concentration of 0.3 ng/ml. Assays were performed in two ways: first, by adding hGH to the BaF-B03 hGHRB2B2 cells, followed by increasing concentrations of mAbs 20 and 21 into replicate wells, and second, by adding either 5 or 50 nM mAbs 20 and 21, followed 15 min later by increasing concentrations of anti-mouse second Ab into replicate wells. Cells were processed as described above for proliferation assays.

c-fos Promoter Assays—Promoter activation assays were performed on CHO-K1 cells in the same manner as described in Refs. 21 and 22) using the c-*fos* 396del219–81 construct (21), except that assay precision was improved by co-transfecting a β-galactosidase construct to normalize for transfection efficiency, and the chosen GHR mAbs were substituted for hGH.

Acute Phase Response Element (APRE) Electrophoretic Mobility Shift Assay (EMSA)—FDC-P1 cells expressing human and rabbit GHRs were grown in RPMI 1640, 10% FCS, and 100 ng/ml hGH. Cells were washed twice with PBS and resuspended in RPMI 1640 supplemented with 0.8% FCS at a concentration of 1.5×10^7 cells/ml. 500-μl aliquots of cells were dispensed into 24-well plates, incubated for 24 h, and then treated with either GH or mAbs at a final concentration of 4.5 or 100 nM, respectively. Following a 15-min exposure to either GH or mAbs, crude nuclear extracts were prepared using the protocol described in Ref. 23.

Each EMSA binding mixture (22 μl) contained 3 μl of nuclear extract (approximately 5 μg of protein), 4 μg of bovine serum albumin, 2 μg of poly(dI-dC), 0.5 μg of labeled oligonucleotide in 12 mM HEPES (pH 7.9), 12% glycerol, 0.12 mM EDTA, 38 mM KCl, 0.9 mM MgCl$_2$, 0.6 mM

TABLE I
Binding and biological characterization of anti-hGHR mAbs

Results are the mean of three determinations with S.E. indicated. The concentration of mAb used was that which gave a maximal response (between 10 and 300 nM).

mAb	Immunogen	Competes with hGH binding domain of			Maximal GH-induced proliferation in FDC-P1 cells expressing			Inhibition of proliferation in FDC-P1 cells expressing		
		G-CSF/hGHR	hGHR	rGHR	G-CSF/hGHR	hGHR	rGHR	G-CSF/hGHR	hGHR	rGHR
					%			%		
1	Rabbit GHR	No	No	Yes	0	0	0	100	40[a]	100
5	Rabbit GHR	No	No	—[b]	0	0	0	100	40	100
7	Rabbit GHR	nxr[c]	nxr	Yes	nxr	0	0	nxr	nxr	100
43	Rabbit GHR	—[d]	—[d]	Yes	56.3 ± 6.7	0	0	50.6 ± 4.3	0	100
44	Rabbit GHR	nxr	nxr	—[b]	nxr	0	0	nxr	nxr	37.6 ± 2.6
263	Rat/rabbit GHR	—[d]	—[d]	—[d]	94.3 ± 5.8	0	0	0	0	10.2 ± 1.7
IC9	Anti-Id hGHR	ND[e]	Yes	ND	3.8 ± 0.5	0	0	43.0 ± 12	28.3 ± 2.0[a]	0
2C8	HumanGHR	ND	Yes	ND	27.7 ± 4.8	0	0	88.0 ± 10.0	100	100
3D2	HumanGHR	ND	Yes	ND	21.8 ± 4.6	0	0	89.5 ± 3.7	100	100
5C6	HumanGHR	ND	Yes	ND	46.6 ± 2.8	0	0	59.0 ± 0	91.1 ± 1.0	93.5 ± 6.6
6C3	HumanGHR	ND	Yes	ND	15.1 ± 1.0	0	0	83.5 ± 2.5	100	100
6F5	HumanGHR	ND	Yes	nxr	8.5 ± 0.8	0	0	100	100	nxr
9H12	HumanGHR	ND	No	ND	44.6 ± 2.3	0	0	0	0	58.0 ± 2.0
10B8	HumanGHR	ND	No	ND	40.3 ± 2.7	0	0	21.1 ± 4.0	0	100

[a] Inhibition has not plateaued by 100 nM mAb.
[b] Shows some inhibition (<50%) of ^{125}I-GH binding, which may be due to inhibition of GHR dimer formation.
[c] nxr, no cross-reactivity of mAb with receptor.
[d] Inhibits GH binding by 50% or less.
[e] ND, not determined.

dithiothreitol, 0.3 mM pefabloc (Boehringer Mannheim, Castle Hill, Australia) and 1.2 μg/ml aprotinin and leupeptin. Samples were incubated on ice for 1 h and electrophoresed on 6% polyacrylamide, 1 × TBE (Tris borate/EDTA) gels. The double-stranded oligonucleotide probe used in EMSA, APREω (5′-GAT CCT TCC GGG AAT TCC TA), contained a high affinity binding site for STAT3 (24). Complementary strands of this probe were synthesized on a Milligen/Biosearch 7500 DNA synthesizer, annealed, 5′-end-labeled with T4 polynucleotide kinase (Pharmacia), and purified on a 10% polyacrylamide gel.

RESULTS

Receptor Expression in Clonal Lines—FDC-P1 cells containing the full-length hGHR (10), rabbit GHR (rbGHR) (8), and chimeric hGHR/mG-CSF receptor (4) have been previously described. The rabbit and human GHR-containing FDC-P1 cells have been shown to possess 212 ± 12 and 372 ± 82 receptors/cell (mean ± S.E.), respectively, when grown in 40 ng/ml hGH. These values are considerably less than that described for the hGHR/mG-CSF containing cells (1000 ± 300 receptors/cell) maintained in GH-free medium (4). Since the number of surface-expressed receptors will influence the likelihood of receptor dimerization by the cross-linking mAbs, FDC-P1 cells expressing GHRs were likewise grown in IL-3 support medium without GH to limit receptor down-regulation. Under these conditions, the receptor expression was shown to increase to 2102 ± 186 receptors/cell and 2393 ± 310 receptors/cell for the hGHR- and rbGHR-containing FDC-P1 lines, respectively.

Transfection of BaF-B03 cells with hGHR and rbGHR resulted in clones expressing between 500 and 15000 receptors/cell in IL-3 support medium. Because we observed that the magnitude of the proliferative response was proportional to the number of expressed receptors (data not shown), we performed proliferation assays on clones expressing approximately the same number of receptors; BaF-B03-hGHRB2B2 has 7419 ± 777 receptors/cell with an affinity of $2.5 ± 0.2 × 10^9$ M^{-1}, and BaF-B03-rbGHR has 7285 ± 618 receptors/cell with an affinity of $6.0 ± 0.6 × 10^9$ M^{-1}. The BaF-B03 clone expressing the loop deleted hGHR has 7540 ± 1190 receptors/cell with an affinity of $1.7 ± 0.4 × 10^9$ M^{-1}.

Precipitation and Inhibition Assays with mAbs—To confirm that the purified mAbs used were active against the human and rabbit GHRs, we determined their ability to precipitate the rabbit and human GHBP and to inhibit the binding of ^{125}I-bGH

to rabbit liver microsomes and ^{125}I-hGH to the hGHR and chimeric (hGHR/mG-CSF) receptor-expressing cells (Table I).

Two of the mAbs, 7 and 44, were unable to precipitate the ^{125}I-hGH·human GHBP complex, although they did react with the rabbit GHR. This result with mAb 7 confirms earlier findings that mAb 7 does not recognize the hGHR (25). All other mAbs recognized the hGHR; however, mAbs 1, 5, 9H12, and 10B8 did not compete with hGH for the GHR binding site. mAb 263 was the only mAb that showed intermediate inhibition of ^{125}I-hGH binding, approximately 50% at a concentration of 100 nM (Table I). This intermediate level of inhibition occurs despite mAb 263 having a high titer for the GHR (25). Against the chimeric receptor, mAbs 43 and 263 are partly effective at displacing hGH binding (Table I).

Against the rbGHR, mAbs 5 and 44 are only partial inhibitors of ^{125}I-bGH binding to the rabbit GHR. These mAbs and mAb 1 have previously been shown to be poor inhibitors of ^{125}-hGH binding to the rbGHR (12–14). We have recently shown (46) that high affinity binding of non-primate GHs to the GHR is dependent on receptor dimerization, so the loss in binding of ^{125}I-bGH observed in the presence of these mAbs may be a result of the inhibition of trimeric complex formation rather than direct competition for the GH binding domain. This is the case for mAb 5 (2).

Cell Proliferation Assays—Because of the large difference in receptor expression observed between FDC-P1 cells grown in either GH or IL-3 (as reported above), the ability of the mAbs to stimulate cell proliferation was tested with cells prepared under both conditions. With the mAbs tested, no significant differences were observed. We have only included in Table I the results obtained with the cells grown in the absence of GH (100 units/ml IL-3), since these cells express the greater receptor number.

Cell Proliferation Assays Performed on FDC-P1 Cells Expressing the Chimeric hGHR/mG-CSF Receptor—FDC-P1 cells containing the chimeric hGHR/mG-CSF receptor were found to proliferate in the presence of a number of the mAbs (examples shown in Fig. 1A). mAb 263 was able to induce the largest response in these cells, achieving a similar maximum response to that obtained with GH alone. When the mAbs were assayed in the presence of a half-maximal dose of GH, a number of
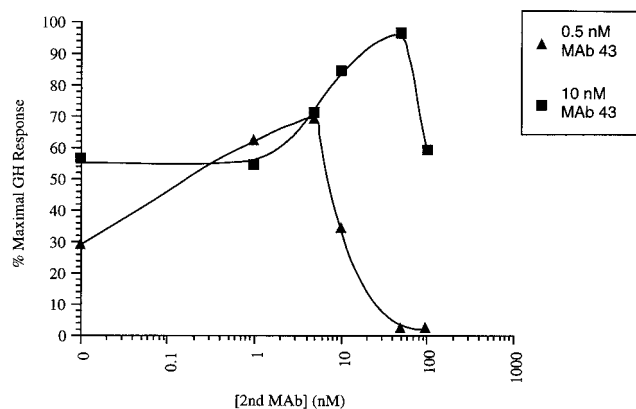
Growth Hormone Receptor Activation

A

B
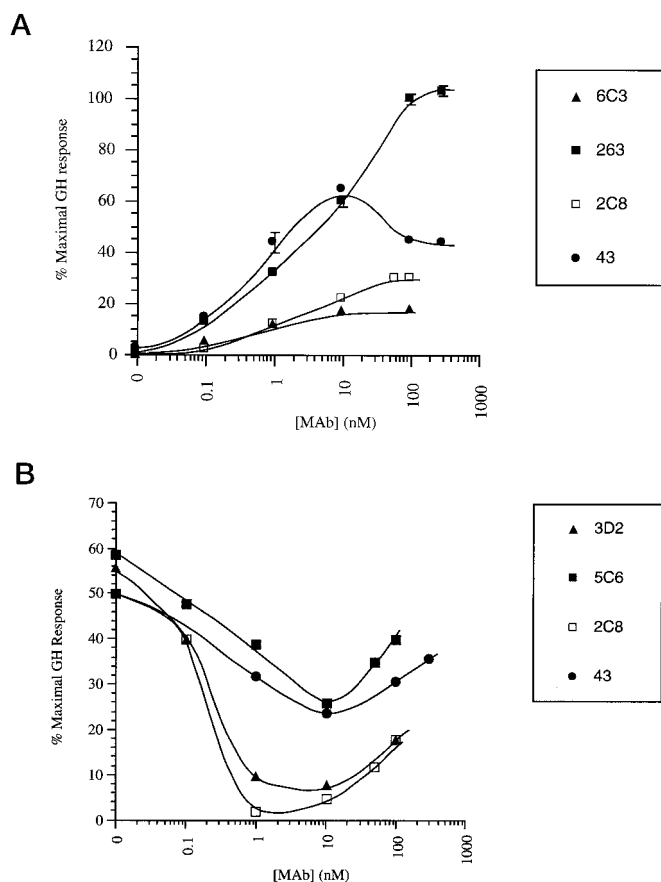
FIG. 1. **Proliferative response of chimeric hGHR/G-CSF-expressing FDC-P1 cells (HC-2 cells) to the presence of mAbs 2C8, 6C3, 7, 43, and 263 only (*A*) and mAbs 2C8, 3D2, 5C6, and 43 in combination with hGH at a final concentration of 2.5 ng/ml (*B*).** Prior to the addition of mAbs, cells were washed in PBS and resuspended in growth media devoid of growth factors (*A*) or in media containing hGH (*B*), as described under "Materials and Methods." Each point is the mean of a quadruplicate determination with S.E. indicated, with the assay having been performed three times. Cell growth was calculated as a percentage of the maximum GH response (typically 0.5 absorbance units at 100 ng/ml), determined in parallel.

mAbs were shown to inhibit the GH-induced proliferation (Table I). In the case of mAbs 1C9, 6C3, 6F5, 1, and 5 this was associated with weak or nil proliferative activity, respectively, when tested without GH present. On the other hand, mAbs 43, 2C8, 3D2, and 5C6 were found to inhibit the GH-stimulated proliferative response, but as the concentration of the mAb was increased, an agonist response was observed, resulting in a U-shaped growth-response curve (Fig. 1*B*).

The maximum proliferative response obtained with the chimeric receptor could be further enhanced by the addition of anti-mouse second antibody. An example is depicted in Fig. 2, where the level of mAb 43 stimulation is increased from 65 to 100% of the GH response when co-incubated with 50 nM second Ab at 10 nM mAb 43 and from 30 to 70% of the GH maximum at 0.5 nM mAb 43 and 5 nM second mAb.

Cell Proliferation Assays Performed on FDC-P1 Cells Expressing rbGHR, hGHR, and Rat GHR—In contrast to the results obtained with the FDC-P1 cells stably expressing chimeric receptor, FDC-P1 cells stably expressing either the full-length hGHR or rbGHR did not yield a proliferative response upon the addition of any of the mAbs (Table I), either by themselves or in the presence of a secondary anti-mouse Ab (results not shown). In fact, a number of the mAbs were inhibitory to the GH-induced cell proliferation. The strongest inhibitors for the hGHR-expressing cells were 2C8, 3D2, 5C6, 6C3,

FIG. 2. **Proliferative response of chimeric hGHR/G-CSF expressing FDC-P1 cells (HC-2 cells) to increasing concentrations of mouse second Ab in the presence of mAb 43, either at 0.5 or 10 nM.** Assays were performed in the manner described under "Materials and Methods," with each point being the mean of a quadruplicate determinations with S.E. indicated and with the assay run on three separate occasions.

and 6F5, while for the rbGHR cells, these same mAbs (excepting 6F5) as well as mAbs 1, 5, 7, and 43 were strong inhibitors of cell proliferation (Table I). In a separate study,[2] the response of FDC-P1 cells stably transfected with rat GHR was examined, and it was found that mAbs 263, 43, 44, and 5 up to 100 nM were unable to elicit a proliferative response, while the hGH-positive control gave a 3.3-fold stimulation.

Cell Proliferation Assays Performed on BaF-B03 Cells Expressing rbGHR, hGHR, and the Loop hGHR Mutant—Because we observed that the BaF-B03 cells expressing the GHR were 10 times more sensitive to GH than FDC-P1 cells expressing the same GHR constructs (the ED_{50} for hGHRFDC-P1 and hGHRBaF-B03 cells is 2.45 ± 0.32 and 0.25 ± 0.02 ng/ml, respectively), we tested our panel of mAbs for their ability to induce a proliferative response in BaF-B03 stable lines. Only two of the mAbs (mAb 263 and anti-Id IC9) were able to elicit a biological response from the hGHR, although this was only a small fraction (approximately 5%) of that with GH alone (Table II). Even in the presence of an anti-mouse second Ab, again only mAbs 263 and IC9 were able to stimulate a proliferative response in the hGHR-expressing BaF-B03 cells (Table II and Fig. 3). This response, however, was considerably less than that seen with GH alone.

With the rGHR-expressing cells, mAb 263 was able to give a stimulatory response that was 23% of that achieved with GH, while no response was observed with anti-hGHR mAb 1C9 (Table II). None of the other mAbs tested were able to induce a proliferative response in either the absence or presence of the secondary Ab.

In contrast to the stimulation seen with mAb 263 against the intact hGHR-expressing BaF-B03 cells, mAb 263 elicited only a weak proliferative response with the BaF-B03 cell line expressing the F′–G′ loop delete mutant hGHR (Fig. 4 and Table II). This was seen although both cell lines displayed the same number of receptors. With this same receptor mutant line, hGH was likewise unable to elicit a maximum response of the order of that seen with wild type hGHR-expressing cells (Fig. 4).

Cell Proliferation Assays Performed on BaF-B03 Cells Expressing hGHR in the Presence of Anti-hGH mAbs 20 and 21—Anti-hGH mAbs 20 and 21 are directed to Ile[36] and Pro[37] (mAb 20 and 21) and Leu[156] (mAb 21) (17), hence away from receptor binding sites 1 or 2 (3) and so should be able to form a quaternary complex. Simultaneous binding of both valencies

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[2] W. Baumbach, unpublished results.

TABLE II

Proliferative responses of mAbs 263 and 1C9 in the presence and absence of a second anti-mouse Fab with BaF-B03 lines

The concentration of mAbs 263 and 1C9 was 100 nM (hGHR B2B2 cells) or 300 nM (rabbit GHR cells) when used without second antibody. Where second (anti-mouse) antibody was added, mAb 263 was used at 50 nM and 1C9 at 100 nM (hGHR B2B2 cells), or 300 nM mAb 263 was used (rabbit GHR cells) with, in all cases, 100 nM second antibody. Results are the mean of three determinations with S.E. indicated.

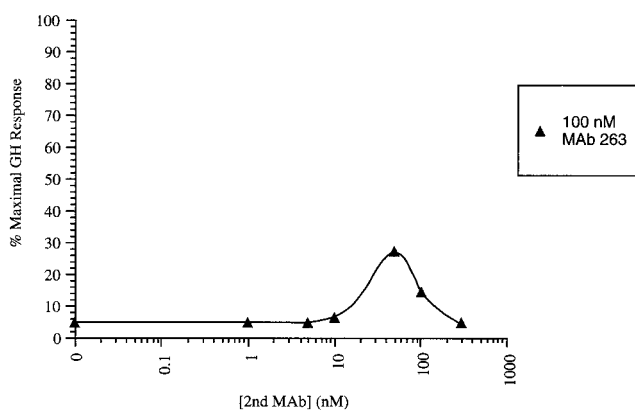| mAb	Maximal GH-induced proliferation in BaF-B03 cells expressing		
	rGHR	hGHR	hGHR loop delete hGHR
	%		
1C9	0	6.4 ± 2.4	ND[a]
1C9 plus 2nd Ab	0	9.5 ± 2.9	ND
263	23.0 ± 4.5	5.4 ± 1.7	4.8 ± 0.5
263 plus 2nd Ab	19.6 ± 2.3	28.2 ± 4.5	6.9 ± 0.6

[a] ND, not determined.

FIG. 3. **Proliferative response of hGHR-expressing BaF-B03 cells in the presence of increasing concentrations of a second anti-mouse Ab in the presence of 100 nM mAb 263.** The assay procedure is described under "Materials and Methods," and each point is the mean of a quadruplicate determinations with S.E. indicated, repeated twice.
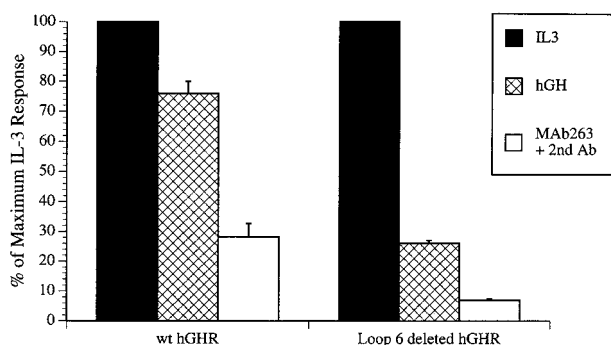

FIG. 4. **Proliferative response of BaF-B03 cells expressing either the full-length hGHR or the F′–G′ loop mutated hGHR to 500 units/ml IL-3, 100 ng/ml hGH, 50 nM mAb 263, or 100 nM mAb 263 co-incubated with 50 nM of anti-mouse Fab.** Assays were performed in the manner described under "Materials and Methods." Each column is the mean of a quadruplicate determination with S.E. indicated. Since BaF-B03 cells are IL-3-dependent, the proliferative responses obtained from the two different GHR cell lines were normalized by expressing the treatment responses as a percentage of the maximum IL-3 response (500 units/ml). Results shown are the mean ± S.E. of quadruplicate determinations in triplicate. *wt*, wild type.

would result in side by side apposition of two GH·(receptor)$_2$ complexes and, if receptor aggregation is a factor in signaling, should result in an enhanced proliferative response. Accordingly, mAbs 20 and 21 were co-incubated with BaF-B03 cells expressing hGHR and a half-maximal concentration of hGH. It was found that neither of these mAbs was able to increase the
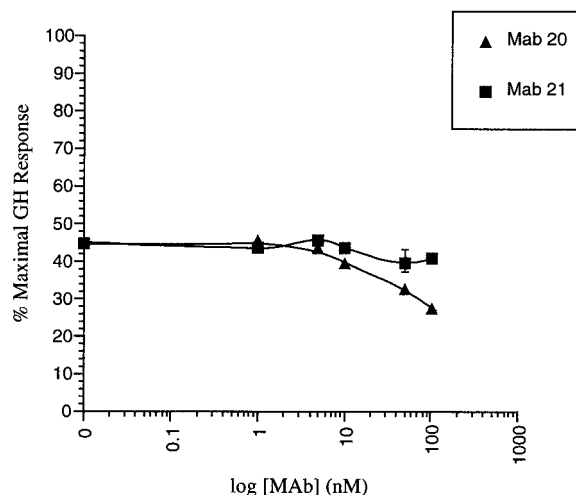

FIG. 5. **Proliferative response of BaF-B03 cells expressing hGHR when assayed in the presence of a half-maximal value of hGH (0.3 ng/ml final concentration) but increasing concentrations of anti-hGH mAbs 20 and 21.** The assay procedure is described under "Materials and Methods," while each point depicted is the mean of a quadruplicate determination with S.E. indicated. Values are expressed as a percentage of the maximum GH response that was obtained at a final hGH concentration of 100 ng/ml.

hGH proliferative response from these cells when used either alone (Fig. 5) or in the presence of a second anti-mouse antibody to further increase receptor aggregation (results not shown).

c-fos Promoter Assays—None of the mAbs tested in the GHR/c-*fos* co-transfection assay in Chinese hamster ovary cells were able to induce a significant luciferase reporter response with either the human or rabbit GHR at 100 nM mAb (examples in Fig. 6). In addition to the assays shown in Fig. 6, a concentration range of 0.1–100 nM of mAbs 43 and 263 were tested against the human GHR (as in Fig. 6*B*). In all cases, the mAbs were unable to induce significant c-*fos* promoter activity, although the positive control hGH was able to give 2.4-fold stimulation.

APRE Gel Shift Assay—Using EMSA, none of the mAbs were able to induce observable induction of binding to an APRE probe with nuclear extracts from FDC-P1 cells expressing either the rabbit GHR (Fig. 7*A*) or human GHR (Fig. 7*B*). In contrast, hGH was able to induce APRE binding in both cell lines.

DISCUSSION

The ability of receptor antibodies to mimic a hormone response is not a new observation. The earliest reports of this phenomenon were with polyclonal antibodies against the insulin and thyrotropin receptors (26, 27). More recent examples include activation of human B lymphocytes via mAbs directed against CD40 (28), stimulation of cell proliferation with a mAb raised against the IL-3 receptor (29), and activation of receptor tyrosine kinase activity with a mAb directed against the epidermal growth factor (30). It is generally believed that bivalent antibodies activate these receptors by inducing dimerization, although in the case of the insulin receptor (which exists as a preassociated dimer (31)), activation as in the example shown by Xiong *et al.* (32) is presumably a consequence of an induced conformational change.

With the GHR, it was shown by Fuh and colleagues (4) that intact mAbs (but not Fab fragments) directed against the extracellular domain of the GHR are capable of stimulating cell proliferation in FDC-P1 cells containing the chimeric hGHR/mG-CSF receptor. This result indicated that signal transduc-
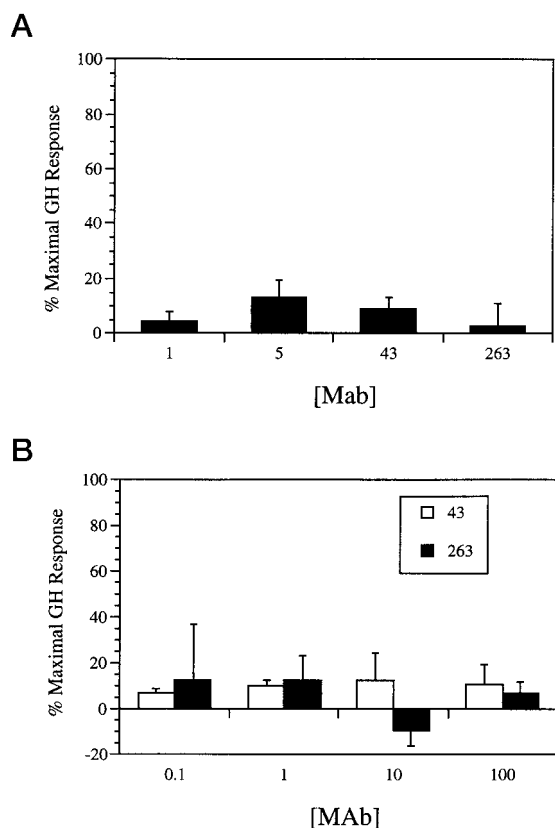
FIG. 6. **c-*fos* promoter transactivation assay showing lack of induction by mAbs.** This assay was undertaken with rbGHR-expressing FDC-P1 cells using the designated mAbs at 100 nM (*A*) or with increasing concentrations of mAb 263 or 43 (*B*) as described under "Materials and Methods." Results shown are the mean ± S.E. of quadruplicate luciferase assays for each point, with the assay repeated twice.

tion through the GHR could be achieved by formation of a GHR dimer, independent of GH. Support for this view came from studies with the prolactin receptor, which is similar to the GHR in both homology (33, 34) and kinetics of binding (35). mAbs to this receptor were found to induce a mitogenic response in Nb2–11C cells, shown by addition of mAbs directed to the extracellular domain (36, 37). Moreover, Fab fragments of the same mAbs were unable to induce a biological response in these studies (36). However, a number of prolactin receptor mAbs were unable to trigger signal transduction (38), and even the mAbs most effective at stimulating proliferation are in excess of 100-fold less potent than prolactin on a molar basis (37).

In order for a mAb to initiate a response through the GHR, the binding epitopes on the dimerized receptor must have a juxtaposed and spatial separation that is complimentary to the distance between the Fab domains in the intact mAb. In addition, the Fab binding sites must have unimpeded access to these epitopes on the receptor. Since the chimeric mG-CSF/hGH receptor has been shown to be activated by intact mAbs raised against the extracellular domain of the GHR, this cell line is appropriate for assessing which of our panel of mAbs are spatially and sterically most likely to correctly dimerize two full-length GHRs and thus to potentially induce a biological response.

The HC-2 FDC-P1 cells, expressing the chimeric hGHR/mG-CSF receptor, were shown to be responsive to a number of mAbs raised against the GHR (Table I and Fig. 1). Of all the mAbs tested, mAb 263 was the most efficient at inducing a proliferative response in these cells, since the maximal response was nearly equivalent to that induced by GH alone
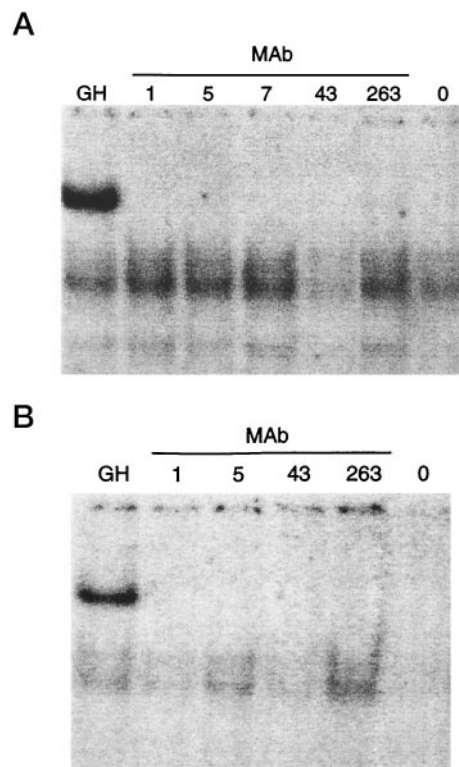
FIG. 7. **STAT3 gel shift (EMSA) showing lack of induction by mAbs.** Nuclear extracts from rbGHR- (*A*) or hGHR- (*B*) expressing FDC-P1 lines exposed to indicated mAbs or hGH for 15 min were run with an end-labeled APRE probe (see "Materials and Methods"). A similarly labeled Oct-1 probe was used as a loading control (23).

(Table I). Four of the mAbs, 43, 5C6, 9H12b, and 10B8 gave a 40% or greater response, while three others, 2C8, 3D2, and 6C3 gave between a 15 and 30% response (Table I). It is notable that there was no correlation between ability to stimulate cell proliferation and competition for the GH binding site on the receptor.

In addition to testing the proliferative capability of these mAbs with the chimeric receptor, we examined each for its ability to inhibit the GH-induced response with this cell line. mAbs 1, 5, and 6F5 were the most potent inhibitors, with mAbs 3D2, 2C8, 6C3, 5C6, 43, 1C9, and 10B8 showing decreasing degrees of inhibitory ability (Table I). From the epitope mapping (Table I), it is not surprising that mAbs 43, 1C9, 2C8, 3D2, 5C6, 6C3, and 6F5 are inhibitory, since they compete with hGH for the GH binding domain. The inability of many of these mAbs to fully abolish the GH-induced signal is most likely due to the stimulatory ability of these mAbs at high concentrations. In support of this theory, a U-shaped dose-response curve was observed with four of these mAbs, 43, 5C6, 3D2, and 2C8 (Fig. 1B). We suggest that these antibodies inhibit hormone stimulation at low concentrations because they block dimerization by hormone, whereas at higher concentrations they bind for a sufficient period to enable the use of both valencies to dimerize and activate the receptor. The inhibitory ability of mAb 5, and possibly mAbs 1 and 6F5, is most likely a result of their sterically impeding dimerization of two GHRs by binding to the GHR dimerization domain or inducing an unproductive conformation for signaling. mAb 5 has previously been shown to inhibit dimerization of the GHBP in solution (2), and the D152H mutation in the dimerization domain of the hGHR has been shown to affect the binding epitope of mAb 5 (39). From these results, it is clear that at least eight of the mAbs are good candidates for inducing a proliferative response in cells con-

taining the full-length GHR, assuming similar steric constraints.

With the FDC-P1 cells containing either the full-length human or rabbit GHRs, none of our panel of 14 mAbs tested were able to stimulate cell proliferation (Table I). In addition, mAbs 5, 43, 44, and 263 were unable to induce a proliferative response in FDC-P1 cells containing the rat GHR receptors (results not shown). A number of the mAbs were, however, potent inhibitors of the GH-induced proliferative response (Table I). This was expected based on their ability to compete with GH for the GHR binding domain, although in the case of mAb 5 (and possibly mAbs 1 and 6F5) this is due to its ability to inhibit dimerization. Consistent with these results, we were unable to show a STAT3/APRE activation when a number of our mAbs were tested (Fig. 7). Further supporting the inability of these mAbs to signal, we found that they were also unable to induce c-*fos* promoter activation in Chinese hamster ovary cells co-transfected with the full-length GHR (Fig. 6).

In the course of our studies with the GHR, we have found that BaF-B03 cells transfected with the GHR are as much as 10 times more sensitive to GH than FDC-P1 cells expressing the same GHR constructs. Accordingly, we tested our panel of mAbs against these GHR-expressing BaF-B03 cells. In contrast to the above results obtained with the FDC-P1 cells, we were able to show that two of the mAbs, 1C9 and 263, are weak activators of cell proliferation in the hGHR-expressing BaF-B03 cells (4.8 ± 0.5 and $5.4 \pm 1.7\%$ of the GH response, respectively), while in the rbGHR-expressing BaF-B03 cells, mAb 263 was able to elicit $23.0 \pm 4.5\%$ of the maximum GH response (Table II). These results suggest that proliferative signaling pathways are better coupled in BaF-B03 cells than in FDC-P1 cells.

Although the affinity of mAb 263 for the GHR is similar to that measured with GH (18), it is possible that the magnitude of the mAb 263 signal may be limited by the instability of the mAb·(GHR)$_2$ complex. Because it has been shown that a second antibody in combination with Fab fragments can be used to promote prolactin receptor signaling (36), we have utilized a second anti-mouse antibody to achieve the same effect. Through use of the chimeric cell line, we were able to show that we could indeed increase the sensitivity and maximum response of the cells to mAb 43 through increasing the concentration of the second antibody (Fig. 2). At high secondary:primary antibody ratios, a decrease in proliferative activity was observed, presumably as a result of the steric hindrance of binding of the primary mAb to the receptor.

In the presence of the second antibody, mAbs 1C9 and 263 were still the only mAbs that were able to produce a proliferative response in the human GHR BaF-B03 line (Figs. 3 and 4 and Table II). For mAb 263, second antibody addition resulted in a response 4-fold greater than achievable with mAb 263 alone, indicating that the secondary mAb is either stabilizing the mAb·(GHR)$_2$ complexes or enhancing GHR oligomerization. Despite the increase in proliferative activity observed with the secondary antibody, the maximum response observed in the hGHR-expressing BaF-B03 cells was still less than one-third of that obtained with GH alone. Indeed, with the rbGHR-BaF-B03 cells, no further enhancement could be achieved, probably because mAb 263 binds with greater than 10-fold higher affinity to the rabbit receptor (14, 25).

In the above experiments with mAb 263 in combination with a second anti-mouse antibody, we could not determine if the greater cell proliferation response we obtained was simply a result of dimer stabilization or due to aggregation of dimerized GHR complexes in a manner that increases the efficiency of signaling (for example by concentrating the number of signal-ing units (*e.g.* JAK2) in a predetermined region of the cell). To address this issue, we utilized mAbs 20 and 21 to induce signaling unit aggregation, because these mAbs have been shown through extensive epitope mapping studies to bind to the nonreceptor interactive region of the hormone, located at the side of the GH·(receptor)$_2$ complex (17). Neither mAb 20 or 21 increased the proliferative response (Fig. 5), and in fact mAb 20 was inhibitory, possibly due to some steric hindrance of GH binding to the GHR. The addition of anti-mouse second antibody to facilitate further aggregation also did not increase the proliferative response. These results, especially that obtained with mAb 21, suggest that aggregation of signaling units does not increase signaling and indicate that the increased proliferation observed through use of the second anti-mouse antibody in combination with the anti-GHR mAbs is a result of stabilization of the dimerized GHR complex.

It is clear from the above results that although it is possible for mAbs raised against the GHR to elicit a proliferation signal with the full-length GHR, this ability is weak and limited to only two of the mAbs we have tested. This limitation exists despite the fact that a number of the mAbs have receptor binding epitopes that are sterically and spatially suited to dimerizing two GHRs that are linked to the fibronectin domains of G-CSF receptor (Table I and Fig. 1). We conclude that the chimeric receptor is not a good model for assessing GH receptor coupling that results from either mAb or GH binding. Indeed, it is clear upon comparing results obtained with mAbs tested against the hGHR/G-CSF and full-length hGHR-expressing FDC-P1 cells that significant differences exist between these receptors. For example, mAb 43 was found to inhibit the GH-induced response in the G-CSF/hGHR FDC-P1 cells, but it is unable to do so in the hGHR FDC-P1 cells. Why is the chimeric receptor more responsive to mAb agonists? Apart from the obvious difference between the GH and G-CSF cytoplasmic domains, it could be that the hGHR/G-CSF receptor exists in a partially activated form or in a form that is more easily able to undergo the transition to the activated receptor. For example, the additional extracellular fibronectin domains in the chimeric receptor could favor facile dimerization. It should, however, be noted that the ability of the antibodies to activate the chimeric receptor is still 100-fold less than hormone on a molar basis, although their affinity for the receptor is similar (12–16, 18).

From the results obtained with the full-length GHR-expressing BaF-B03 cells and mAbs 1C9 and 263, it is apparent that although these mAbs have the ability to stimulate a proliferative response, the magnitude of this response is significantly lower than that observed with GH alone even with the help of a secondary Ab. The inability of mAbs to evoke a response with a full-length receptor equivalent to that obtained with the ligand alone is also seen with the prolactin receptor (36, 37) and erythropoietin receptor (40). It would therefore seem that although mAbs are able to induce dimerization of receptors, the efficiency of signal activation is reduced. This could be either a result of critical steric requirements for alignment of dimerization domain residues in domain 2 (5) or a requirement for a key conformational change that allows efficient coupling to second messenger pathways.

What is the evidence that a conformational change plays a role in signaling through the single transmembrane spanning segment? First, in the crystal structure, the angle between domains 1 and 2 differs between receptor 1 and 2 by 9° (3). Second, JAK2 binding to the GHR is weak until hormone binds (41),[3] suggesting that a hormone-induced conformational

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[3] C. Carter-Su, personal communication.

change increases the availability of box 1. Third, Mellado *et al.* (42) have recently presented evidence based on an agonist GHR mAb directed to the link (hinge) region between domains 1 and 2 that this mAb induces a conformational change required for GHR signaling. Fourth, hormone binding loops exist within the crystal structure of the GHR subunits 1 and 2, which differ in conformation between receptor 1 and 2 as a result of hormone-induced assembly of the signaling complex (residues 101–106, 163–168 (3), and 218–221 (8)). The β-loop connecting the F'–G' strands in domain 2 (residues 216–220) is adjacent to the WS*X*WS box equivalent in the GHR (YGEFS) and the link (hinge) region and is also the last turn prior to insertion into the membrane. We have proposed that a GH-induced conformational change in this loop is necessary for proliferative signaling, since GH mutants interacting with this loop appear to act as partial antagonists for proliferation (8). Moreover, removal of residues on this β-loop strikingly reduces proliferative signaling in FDC-P1 cells expressing these mutant receptors (7). Accordingly, hormone-induced conformational changes in this region appear to be components of the mitogenic signal.

In support of the importance of this F'–G' loop in signaling, we have shown here that, like GH itself, mAb 263 is unable to induce a wild type receptor response in BaF-B03 cells expressing the loop delete receptor (Table II and Fig. 4). Since this cell line expresses the same number of receptors as the wild type control cell line that responds to mAb 263 and since we have shown that the introduced mutation does not adversely affect binding of mAb 263 to this receptor (indeed, binding affinity is increased 2-fold), we conclude that this loop is also a necessary component in mAb 263 signaling. This would suggest that mAb 263 is not only dimerizing two GHRs but also induces the same conformational changes required for signal transduction as GH itself.

This study does not rule out the possibility that fully effective mAb agonists for the GH receptor exist or can be engineered. Indeed, Wang *et al.* (43) have reported a mAb to the GHR that is able to stimulate growth in the hypophysectomized rat. A similar argument applies to small GH mimetics. Indeed, peptide mimetics have been constructed that are able to both dimerize and activate the homologous erythropoietin receptor (44). Of note, the crystal structure of the peptide mimic complexed with two erythropoietin receptors reveals that the F'–G' loop, which we have shown to be important in GHR signaling (7), appears to be one of the major peptide binding epitopes on the receptor (44).

We conclude from this study that particular mAbs against the GHR are able to activate signal transduction through the full-length human and rabbit GHRs, although, in the case of the two mAbs (one an anti-idiotype hGH mimetic) identified here, the efficiency of signaling is significantly reduced compared with that obtained with GH alone. This could be a result of particular steric constraints for the membrane-bound receptor or a requirement for a specific conformational change. Since our data indicate that the receptor epitope involved in binding of the most effective agonist mAb is involved in a conformational change, we propose that in addition to dimerization, specific conformational changes within the receptor are required for effective signaling.

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Activation of Chimeric and Full-length Growth Hormone Receptors by Growth Hormone Receptor Monoclonal Antibodies: A SPECIFIC CONFORMATIONAL CHANGE MAY BE REQUIRED FOR FULL-LENGTH RECEPTOR SIGNALING

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