Growth hormone (GH)-independent Dimerization of GH Receptor by a Leucine Zipper Results in Constitutive Activation*

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Growth hormone initiates signaling by inducing homodimerization of two GH receptors. Here, we have sought to determine whether constitutively active receptor can be created in the absence of the extracellular domain by substituting it with high affinity leucine zippers to create dimers of the growth hormone receptor (GHR) signaling domain. The entire extracellular domain of the GHR was replaced by the hemagglutinin-tagged zipper sequence of either the c-Fos or c-Jun transcription factor (termed Fos-GHR and Jun-GHR, respectively). Transient transfection of Fos-GHR or Jun-GHR resulted in activation of the serine protease inhibitor 2.1 promoter in Chinese hamster ovary-K1 cells to a level equal to that achieved by fully activated wild type GHR. Furthermore, stable expression of Jun-GHR alone or Fos-GHR and Jun-GHR together in the interleukin 3-dependent BaF-B03 cell line resulted in cell proliferation after interleukin 3 withdrawal at a rate equal to maximally stimulated wild type GHR-expressing cells. Activation of STAT 5b was also observed in Fos-Jun-GHR-expressing cells at a level equal to that in chronically GH-treated GHR-expressing cells. Thus, forced dimerization of the transmembrane and cytoplasmic domains of the GHR in the absence of the extracellular domain can lead to the constitutive activation of known GH signaling end points, supporting the view that proximity of Janus kinase 2 (JAK2) kinases is the essential element in signaling. Such constitutively active GH receptors may have particular utility for transgenic livestock applications.

Growth hormone (GH) is a potent regulator of somatic growth and a broad spectrum of metabolic processes. Its wide variety of effects on target cells are mediated by a single membrane-spanning receptor (GHR), which is expressed in over 40 different tissues (reviewed in Ref. 2). Early studies showed that GH binds via a high affinity interaction with one GHR receptor, followed by a lower affinity interaction with the second identical GHR forming a GH(GHR)2 trimeric complex (3). Formation of this complex is the first step in signal transduction, which is presumed to bring together and activate two JAK2 molecules, each of which associates with a highly conserved membrane proximal proline rich region in the cytoplasmic domain referred to as box 1 (4). Activation of JAK2 results in activation of signaling molecules, such as STATs, MAP kinases, phosphatidylinositol 3'-phosphate kinase, and protein kinase C, and elevates intracellular calcium, leading to the transcriptional regulation of many genes (4).

The biological role of GHR dimerization was first studied by Fuh et al. (5) using a cell line that expressed a chimeric receptor consisting of the binding domain of GHR fused to the extracellular fibronectin and cytoplasmic domains of the G-CSFR. These workers showed that wild type GH could trigger proliferation in these cells, although a GH analogue with an unfavourable substitution in the low affinity site 2 domain was unable to cause proliferation, because it was unable to induce GHR dimerization. These workers also reported that monoclonal antibodies to the GHR were able to elicit a full proliferative response in these cells, presumably by dimerizing the chimeric receptor in a fashion analogous to GH. More recently, we have studied the importance of interreceptor interactions by creating mutations in the dimerization domain, the only contact domain between the two receptor sub-units (6). We found that charge reversal mutagenesis severely reduced the ability of the receptor to signal, presumably by preventing trimeric complex formation. Thus, there is strong support for the essential role of dimerization in GHR signal transduction, with alignment of the two receptors being set by bonding interactions across the dimerization domain.

Given this view, dimerization of receptor subunits by means of bivalent antibodies should result in signal generation. However, although monoclonal antibodies to the extracellular domain of the GH receptor are able to generate full proliferative responses in FDCP-1 lines expressing a chimera of the GH receptor (extracellular) fused to the G-CSFR (4, 7), these same antibodies are unable to support proliferation of FDCP-1 cells expressing full-length GH receptor (7). Thus, of 15 anti-GHR mAbs examined, none could induce proliferation in FDC-P1 cells expressing the full-length GH, whereas 8 could induce proliferation in the FDC-P1 cells expressing the G-CSFR chimera used by Fuh et al. (5). Two mAbs could cause the more...
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Sensitive hGHR-BaF-B03 cells to proliferate, but they did so very weakly (maximum 23% of GH level). These results raise the possibility that dimerization of the GHR alone is not sufficient for its activation and raise questions about its mode of action, including the possible requirement for a specific hormone-induced conformational change for signaling (see discussion in Ref. 7), a view supported by another recent study using monoclonal antibodies to the GH receptor (8). Very recently a crystallographic study of the closely homologous erythropoietin receptor has shown that it can exist as a preformed dimer by virtue of inter receptor interactions located in the same loops of the receptor that otherwise bind the hormone (9). Furthermore, this preformed dimer places the membrane entry points of the extracellular domain around 73 Å apart, whereas the hormone bound receptor dimer has entry points around 39 Å apart (9). Using an elegant fragment complementation assay, Remy et al. (10) presented evidence that a conformational change results from hormone binding, which brings the membrane entry points close together, paralleling the subunit relationships seen with the crystal structures of the unbound and ligand occupied receptor dimers. This new paradigm for cytokine receptor signaling could explain the difficulty in obtaining agonist responses with monoclonal antibodies to the GH receptor, because only a small subset would be able to trigger the conformational changes necessary to bring the membrane proximal sequences into proximity. Indeed, a similar study performed with the EPO receptor found that only 4 of 96 monoclonal antibodies to the extracellular domain were able to activate the receptor (11).

Whether the original sequential dimerization model or the preformed dimer/conformational change model applies to the GH receptor, it should be possible to create constitutively active GH receptors if the receptor subunits are correctly aligned in appropriate proximity. This study seeks to establish whether the original sequential dimerization model or the preformed dimer/conformational change model applies to the GH receptor.

EXPERIMENTAL PROCEDURES

Expression Plasmid Construction

Polymerase chain reaction was used to amplify the cDNA encoding the 27 amino acid signal peptide from porcine GHR (pGHR) such that the product included a 5′ HindIII restriction site and a 3′ BamHI restriction site (Fig. 1). This fragment was then cloned into the expression vector pcDNA3.1+ (Invitrogen). Synthetic oligonucleotides encoding the hemagglutinin (HA) tag (YPYDVPDYAS) (12) were annealed and cloned into the pcDNA 3.1+ vector (contains the neomycin resistance gene) downstream of the pGHR signal peptide sequence at BamHI and EcoRI sites. Synthetic oligonucleotides encoding either the mouse c-Fos leucine zipper (amino acids 162–200) or the mouse c-Jun leucine zipper (amino acids 277–315) that contained a 5′ EcoRI site and a 3′ NotI site were annealed and cloned into the vector downstream of the HA tag. Polymerase chain reaction was used to amplify the GHR transmembrane and cytoplasmic domains (amino acids 251–638) such that the product included a 5′ NotI and a 3′ XhoI overhang that was then cloned downstream of the leucine zipper. The entire insert was sequenced and found to contain no errors. Eleven residues of receptor sequence were left at the membrane proximal end of the domain between the zipper sequence and the transmembrane sequence to allow a degree of flexibility similar to that of wild type GHR. This new paradigm for cytokine receptor signaling could explain the difficulty in obtaining agonist responses with monoclonal antibodies to the GH receptor, because only a small subset would be able to trigger the conformational changes necessary to bring the membrane proximal sequences into proximity. Indeed, a similar study performed with the EPO receptor found that only 4 of 96 monoclonal antibodies to the extracellular domain were able to activate the receptor (11).

CHO-K1 Cell Culture, Transfection, and Spi 2.1-Chloramphenicol Acetyltransferase (CAT) Assay

This was performed as described in Hansen et al. (13). Briefly, cells were transfected with 3 μg of plasmid containing the β-galactosidase gene, 1.5 μg of Spi 2.1-CAT reporter construct, and varying amounts of chimeric receptor plasmid using the calcium phosphate method. After 48 h, cells were lysed, and extracts were normalized for β-galactosidase activity before the CAT assay was performed. As a control, 1.5 μg of pGHR receptor expression construct was transfected in place of the chimera constructs, and cells were stimulated with 20 nM hGH.

BaF-B03 Cell Culture and Transfection

BaF-B03 cells (gift from Dr Tom Gonda, Hanson Institute, Adelaide, Australia) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells at 50% confluency in a 10-cm dish (1 day after plating) were transfected by calcium phosphate procedure: DNA-precipitate was made by mixing 500 μl of 0.25 M CaCl2 with 20 μg of eukaryotic expression vector followed by dropwise addition to 500 μl of 2× HBSS (50 mM N,N-bis(2-hydroxyethyl)-2-aminosethanesulfonic acid, 280 mM NaCl, 1.5 mM Na2HPO4, pH 6.9). The precipitate was left for 15 min and subsequently added dropwise to cells remaining in the medium, followed by 15 ml of medium then incubation in a 5% CO2 humidified atmosphere containing 5% carbon dioxide. The next day, the medium was changed, and cells were removed to a humidified atmosphere containing 5% carbon dioxide for a further 24 h.

Cell Proliferation Assay

Cells were suspended in RPMI medium supplemented with 0.5% serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cell suspensions were plated at a limiting dilution and single cells were isolated and seeded into their respective selection media. In the case of each of the pGHR, pE, and Spi plasmid pGHR plasmid constructs, cells were transduced with a genetic resistance plasmid, followed by selection with 1.5 mg/ml G418 for at least 4 days. pGHR-expressing cells were characterized for receptor expression by 125I-hGH binding assay and Scatchard analysis as described in Ref. 14. This involved growing the cells in IL-3 (40 ng/ml) and 10% serum supplemented with 20 μg/ml of 125I-hGH, and then incubating them overnight at 4°C in isonicotinic glucose binding buffer with labeled hGH in the presence of increasing concentrations of hGH (14).
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Electrophoretic Mobility Shift Assay

1 × 10³ BaF-B03 cells were washed with PBS, resuspended in RPMI medium supplemented with 0.5% serum supernate, and then incubated for 8 h at 37 °C. Cells were harvested by centrifugation, and nuclear extracts were prepared as described in Ref. 17, except for pGHR-expressing cells, which were treated with 4.5 mg hGH for 20 min or 16 h prior to harvesting. Electrophoretic mobility shift assay was performed as described in Ref. 7 except that aprtein, leupeptin, and Pefabloc were replaced with Complete inhibitor (Roche Molecular Biochemicals), and the oligonucleotide probe contained the consensus binding site for STAT 5 (AGATTCTAGAATTTCAATCC, Santa Cruz Biotechnology, sc-2565). Supershift experiments were performed by adding 1 μg of either STAT 5a antibody (Santa Cruz Biotechnology, sc-1081) or STAT 5b antibody (Santa Cruz Biotechnology, sc-835) to the reaction after the addition of probe and incubating the mixture for 1 h on ice.

RESULTS

Construction of Expression Plasmids—Two chimeric cDNA molecules were made such that when co-expressed, two identical cytoplasmic domains of the GHR are brought into close proximity (see Fig. 1). Each chimera contains the leucine zipper sequence from either c-Fos or c-Jun in place of almost the entire porcine GHR extracellular domain sequence as well as the HA tag at the amino terminus of the mature protein. A control construct lacking the zipper sequence was also made.

Dimerization of Fos-GHR and Jun-GHR—To test the ability of Fos-GHR and Jun-GHR to dimerize, HEK 293 cells (known for high level expression) were transiently transfected with each expression construct and the chimeric protein was analyzed by Western blot. Fig. 2 shows that both Fos-GHR and Jun-GHR can homodimerize and that this dimer is stable enough to withstand denaturing conditions in the presence of reductant.

Activation of the Spi 2.1 Promoter by Fos-GHR and Jun-GHR in CHO-K1 Cells—As an initial test for activity of the chimera, the constructs were transiently expressed in CHO-K1 cells along with a reporter construct consisting of the Spi 2.1 promoter linked to the CAT reporter gene (13). Fig. 3 shows that increasing amounts of transfected chimeric plasmids, together or separately, result in increasing levels of reporter gene activity, to a point that is equivalent to the maximum amount achieved by full-length GHR triggered with GH (20 ng hGH). It should be noted that the Fos-GHR chimera was just as capable of activating the reporter gene as the Jun-GHR chimera, indicating that productive Fos-GHR homodimers are forming (see below). Specificity in signaling is apparent, because no signal was seen by the chimera when all of its cytoplasmic tyrosine residues were mutated to phenylalanine, which accords with the requirement for STAT 5 docking through its SH2 domain for activation (4, 13). In order to demonstrate the functional role of the Fos/Jun zippers, a construct without the zipper coding sequence, but otherwise identical, was included in the analysis. This construct was able to produce some constitutive activation of the Spi 2.1 promoter, although equivalent activation to that produced by Fos or Jun-GHR required around 75-fold higher concentration of this expression construct (Fig. 3).

Creation of Stable BaF-B03 Cells Lines—The IL-3-dependent pro B cell line BaF-B03 has been previously shown to respond to very low levels of GH when stably transfected with human and rabbit GHR (7, 18). The sensitivity of BaF-B03 cells to GH makes them an ideal system to test the signaling ability of the Fos-GHR and Jun-GHR chimeras in a physiologically relevant way. Transfection of full-length porcine GHR and selection with GH yielded colonies that were cloned by limiting dilution and left on ice for 1 h. For chimeras expressing cells, anti-HA antibody used for probing was clone 12CA5, not 3F10 (Roche Molecular Biochemicals).
plasmid alone or co-transfected with Fos-GHR plasmid, cytokine-independent cell proliferation was observed, and these cells were cloned by limiting dilution (named Jun-BaF and Fos/Jun-BaF, respectively). However, despite several attempts, no colonies were evident in populations transfected with Fos-GHR alone. Transfection efficacy was confirmed in these populations by performing a parallel selection with G418 that yielded numerous colonies with IL-3 support.

**Expression of Fos-GHR and Jun-GHR Chimeras in BaF-B03 Cells**—Immunoprecipitation and blotting demonstrated correct expression of the chimeric and full-length receptors (Fig. 4). In the case of Fos-GHR, G418-resistant populations were used to confirm expression of the chimera because no constitutively proliferating colonies were obtained. Despite the fact that Jun-GHR homodimers and Fos-GHR/Jun-GHR heterodimers can cause proliferation, Western blotting was unable to show the formation of the dimeric complex in BaF-B03 cells.

In order to estimate the relative expression levels of wild type and Jun-GHR proteins, a number of lines expressing Jun-GHR were compared with the line expressing 3457 6 3466 wild type pGHR surface receptors per cell by immunoblot. In this comparison, solubilized cell extracts of 1 3 10^8 cells were immunoprecipitated with one antibody to the cytoplasmic domain of the receptor, and probed with another (Fig. 4A). Densitometric analysis provided estimates of 81, 107, 225, and 249% of wild type pGHR expression for four Jun GHR clones, and the thymidine incorporation rates of these clones were 67 6 1, 76 6 0.8, 106 6 2, and 141 6 1%, respectively, those of wild type pGHR (see below). It should be noted that the level of expression of zip-GHR constructs (as determined by immunoblot as above) was vastly greater in HEK 293 cells and CHO-K1 cells transiently transfected with zip-GHR expression constructs (results not shown).

**Surface Expression of Fos-GHR and Jun-GHR in BaF-B03 Cells**—Fluorescence activated cell sorting using the anti-HA antibody was performed on Fos/Jun-BaF clones to measure surface expression. Even at high antibody concentrations, very
low cell fluorescence was detected in the clones expressing the chimeras (Fig. 5A). This level of fluorescence is very similar to that found when pGHR-BaF cells are treated with 20 nM hGH for 16 h, a stimulus that is known to down-regulate the receptor by a factor of 10 (Fig. 5B) (14). It appears that because the chimeric receptor is constitutively active, it is constantly in a down-regulated state.

### Proliferation Rates of Clones Expressing Chimeric Receptors—

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and [3H]thymidine incorporation assays were used to demonstrate that proliferation rates of Jun-BaF and Fos/Jun-BaF clones are almost identical to that achieved by pGHR-BaF when stimulated with a maximal dose of GH (20 nM, Fig. 6). Rates have been normalized to each clone’s ability to proliferate in response to a maximal dose of IL-3, which corrects for intrinsic differences in the ability of clones to proliferate. In the case of the pGH receptor-expressing clone, this was 54 ± 2.6% (n = 10) of the maximal IL-3 response.

### STAT 5 Induction by Clones Expressing Chimeric Receptors—

STAT 5 is a latent transcription factor that is phosphorylated by JAK2 in response to receptor activation and is known to bind a specific cis element in response to GH. Fig. 7A shows that the amount of STAT 5 that is able to bind DNA in the Fos/Jun-BaF clones is equivalent to that in pGHR-BaF cells that have been treated for 16 h with 20 nM GH. As determined by band densitometry, this level of activity for the construct lacking a zipper domain is shown for the 0.02, 0.1, and 1.5 μg amounts. Results shown are the mean ± S.E. of three independent experiments.

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**Fig. 3. Spi2.1-CAT reporter gene activity of Fos-GHR (open columns), Jun-GHR (striped columns), Fos-GHR plus Jun-GHR (black columns), and minus zipper (cross-hatched columns) chimeras expressed in CHO-K1 cells.** Increasing amounts of expression plasmid were transiently transfected and cells assayed as described under “Experimental Procedures.” Micrograms shown for Fos-GHR plus Jun-GHR co-transfections are totals comprising equal amounts of each plasmid. Activity is expressed as a percentage of maximally stimulated wild type pGHR-expressing cells transfected with 1.5 μg of plasmid and stimulated with 20 nM hGH. The far right columns (No Tyr) show the level of activity of chimeras that have all intracellular tyrosines mutated to phenylalanine (1.5 μg transfected). The level of activity for the construct lacking a zipper domain is shown for the 0.02, 0.1, and 1.5 μg amounts. Results shown are the mean ± S.E. of three independent experiments.

**Fig. 4. Immunoblot of Fos-GHR and Jun-GHR expressed in BaF-B03 cells.** Immunoprecipitation and immunoblot were performed as described under “Experimental Procedures,” using anti-rabbit GHR cytoplasmic domain for immunoprecipitation and HA tag antibody as probe, except for A, in which case the rat GHR cytoplasmic antiserum was used for immunoprecipitation and rabbit GHR cytoplasmic antiserum was used as probe, in order to facilitate comparison of expression level for Jun-GHR and wild type pGHR. A, lanes 1–4, different clones of Jun-GHR (lanes 1 and 2, high level expression; lanes 3 and 4, low level expression); lane 5, wild type pGHR. B, lane 1, untransfected cells; lanes 2–4, constitutively proliferating clones 1–3 expressing both Fos-GHR and Jun-GHR together. C, lane 1, untransfected cells; lanes 2 and 3, two independently transfected G418 resistant populations expressing Fos-GHR. D, lane 1, untransfected cells; lanes 2–4, constitutively proliferating clones 1–3 expressing Jun-GHR. Molecular weights are indicated on the left (×10−3). Images shown are representative of at least two independent experiments.
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A central problem in class 1 cytokine receptor signaling is defining the means used by the hormone to induce proximity of the Janus kinase molecules bound to the membrane proximal box 1 sequence in the cytoplasmic domain. Proximity of the JAKs induces transphosphorylation and activation, initiating a variety of signaling pathways. The work presented here shows that appropriate dimerization of cytoplasmic domains is all that is required to generate a fully active receptor.

Because we were unable to fully activate the receptor through the use of bivalent antibodies, we have used leucine zipper/GHR chimeras that dimerize in the absence of ligand without the extracellular hormone binding domain. We chose this approach because such zippers are likely to form closely apposed dimers, thus avoiding the possibility that steric constraints related to mAb size are preventing the close association of the GHR cytoplasmic domains. Crystal structures of Fos-Jun zippers (19) show that the α-helices are approximately 9 Å apart at their C termini, suggesting that the Fos-GHR and Jun-GHR chimeras would be similarly spaced in the membrane. This distance is closer than the 29 Å distance between the most membrane proximal residue of the receptor resolved in the 2:1 complex (i.e. between the α-carbons of proline 234) based on both the DeVos et al. (20) structure and the Sundstrom et al. (21) structure. However, the presence of an 11-residue residual receptor sequence just above the membrane, linking the remainder of the receptor to the leucine zippers, may introduce an element of flexibility in the alignment of box 1. Our modeling of this linker region and the transmembrane domain indicates the existence of a β-turn that may help to position the α-helical transmembrane domains more closely together. Whatever the role of this 11-residue linker, attachment of the zippers to it has allowed us to mimic the placement of the engaged dimerization domain as well as avoiding any problems that may arise from situating the zipper too close to the cell surface (e.g. phospholipid headgroup interference). The finding that a dimerized GHR lacking its extracellular domain can signal effectively does not preclude the possibility that the extracellular domain of the full-length GHR undergoes a local conformational change in response to GH binding that is necessary for proper alignment of the intracellular signaling domains. Such a conformational change could be analogous to that proposed for the predimerized erythropoietin receptor, discussed in the Introduction (9, 10), which must result in correct apposition (alignment) of the bound JAK2 molecules for effective signaling. This is demonstrated by the crystal structures of small molecule agonists and antagonists of the EPO receptor, which show different angular displacements of the receptor subunits (22, 23). There is evidence that binding of JAK2 to the GHR receptor increases as a result of hormone binding (24), which could be the result of a conformational change in the box 1 region or of additional interactions between bound JAK2 molecules brought about by their apposition. JAK2 can be activated through dimerization, as demonstrated by use of a GHR receptor-JAK2 chimera with the cytoplasmic domain substituted by JAK2 (25). This enabled activation of JAK2 on addition of hormone (presumably by transphosphorylation), suggesting that proximity rather than precise alignment is required.

Co-transfection of CHO-K1 cells with the Spi 2.1-CAT reporter gene and increasing amounts of Fos-GHR or Jun-GHR cDNAs resulted in a level of promoter activity equivalent to that achieved by a maximally activated wild type GHR (see Fig. 3). This implies that the chimeras are able to dimerize and activate JAK2 normally and also to activate STAT 5 because this signaling molecule has been shown to bind a specific element in the Spi 2.1 promoter in response to GH (26). Mutation of all the intracellular tyrosines to phenylalanine prevented both chimeras from signaling, demonstrating that the signaling seen with the Fos-GHR and Jun GHR is originating from the GHR cytoplasmic domain and is not an artifact of over expression of leucine zippers, for example by sequestering endogenous c-Fos or c-Jun. It has been reported that, in solution, Fos zippers form only weak homodimers, although they form strong heterodimers with Jun zippers, whereas Jun zippers can also form reasonably strong homodimers (12). It was therefore surprising to find that Fos-GHR was able to activate the Spi

Fig. 5. Representative fluorescence-activated cell sorting profile of BaF-B03 clones expressing Fos-GHR and Jun-GHR together. Immunostaining and cell sorting were performed as described under "Experimental Procedures." A, dotted line, untransfected cells; boldface line, BaF-B03 cells expressing wild type GHR after 16 h of GH treatment using mAb 263 (17 mg/ml) as probe. B, dotted line, untransfected cells; boldface line, constitutively proliferating BaF-B03 cells expressing Fos-GHR and Jun-GHR together, using 17 mg/ml HA antibody as probe. Data shown are representative of at least three independent experiments performed on three separately isolated clones.

because it is supershifted by the addition of anti-STAT 5b antibody to the reaction mixture before gel loading (Fig. 7B).
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FIG. 6. Constitutive proliferation of BaF-B03 cells stably expressing Jun-GHR (open columns) and Fos-GHR plus Jun-GHR together (black columns). Proliferation levels (as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye incorporation) of three independently isolated clones were measured as described under “Experimental Procedures.” Results are shown as a percentage of a wild type pGHR-expressing clone maximally stimulated with 4.5 nM GH after being normalized to the maximal proliferation rate of each clone in response to IL-3. Untreated wild type pGHR-expressing cells were used to measure background for constitutively proliferating clones. Results shown are the mean ± S.E. of at least three independent experiments. All results were confirmed with [3H]thymidine incorporation assay.

2.1-CAT reporter equally as well as the Jun-GHR chimera, indicating equivalent Fos-GHR homodimer formation. In order to explain this result, it is important to remember that two-dimensional interactions in the plane of the cell membrane are considerably more favorable than those in solution (27). Although the tendency for Fos-GHR to homodimerize would be weak in solution (Kd 6 μM (12)), such dimers are likely to be stabilized when aligned in the cell membrane. In support of this, Patel et al. (28) showed that a chimera of the Fos zipper sequence fused to the granulocyte-macrophage colony-stimulating factor receptor was able to form a disulfide-bridged dimer if a free cysteine is added near its amino terminus. This suggests that the Fos zipper sequence is capable of forming homodimers to some degree, and because the level of expression in CHO-K1 cells is high (relative to that in BaF cells), it is likely that the excess receptor forces the dimerization equilibrium toward homodimer formation. Furthermore, in a cell that expresses a greater number of receptors than effector molecules such as JAK2 (i.e. “spare receptors”), only a relatively small proportion of those receptors is required to yield a maximal response from the cell (29). Therefore, in the case of transient transfection of CHO-K1 cells, it appears that the weak tendency for Fos-GHR to homodimerize is sufficient to enable coupling of dimers to all the available effector molecules, resulting in a maximal response. Indeed, as we show in Fig. 3, even in the absence of a zipper sequence, there is some constitutive activity, although it requires around 75-fold higher concentration of expression plasmid. This may be a result of the high level of expression in CHO cells, or there may be weak interreceptor interactions between transmembrane and/or cytoplasmic domains.

In contrast to the transiently transfected CHO-K1 cells, BaF-B03 cells that were stably transfected with Fos-GHR were unable to proliferate upon the withdrawal of IL-3 despite several transfections. Expression was confirmed by Western blotting of cells that were selected in parallel with G418 (see Fig. 4C). Based on immunoblot, the level of stable expression of the chimeras in BaF-B03 cells is far lower than that seen in CHO-K1 cells, so that the dimerization equilibrium will favor unproductive monomers. Alternatively, it is possible that the weak, short lived homodimerization of Fos-GHR is insufficient to recruit the signaling molecules required for proliferation in a BaF-B03 cell, whereas it is sufficient to recruit JAK2 and STAT 5 in a CHO-K1 cell. When the Fos-GHR and Jun-GHR constructs were co-transfected, IL-3 withdrawal yielded constitutively proliferating colonies. Clones from these colonies proliferated at levels similar to pGHR-BaF cells that were maximally stimulated with GH (see Fig. 6). Similar results were obtained when Jun-GHR was transfected alone, confirming previous work showing that Fos and Jun zippers form strong heterodimers and Jun can form strong homodimers. Comparative immunoblot analysis (Fig. 4A) indicates that the Jun-GHR chimeras and the wild type pGHR are expressed at similar level, which implies that the chimera is functioning with similar efficiency to the wild type receptor.

Based on the fact that the Spi 2.1 promoter was activated by the chimeras in CHO-K1 cells, it was thought that STAT 5 would be up-regulated in Fos/Jun-BaF cells. EMSA showed
that STAT 5 (β isofrom, see Fig. 7B) is indeed activated and binds its consensus sequence in these cells to a degree that is similar to chronically GH-treated pGHR-BaF cells (Fig. 7A). This level of induction is 6-fold lower than that achieved after 20 min of maximal GH treatment, presumably due to a combination of receptor down-regulation and induction of negative regulatory proteins such as SOCS-3 (suppressor of cytokine signaling 3) (30). This result is consistent with the notion that the Fos-GHR/Jun-GHR heterodimer is constitutively active and is down-regulated in a manner similar to wild type GHR in the presence of 20 nM GH. This view is supported by the fluorescence-activated cell sorting data of Fig. 5.

In conclusion, we have been able to generate constitutively active GH receptors by, in effect, substituting the dimerization domain with a leucine zipper. In order to rationalize this result with our previous study using mAbs to the receptor (7), which suggested that a hormone-induced conformational change may be necessary for signaling, and the results of Wilson’s group (9, 10), we propose that GH (or the appropriate mAb) induces a conformational change in the extracellular domain that is necessary for optimum alignment and bonding of the dimerization domain residues. This stabilizes the receptor dimer in a conformation that holds the box 1 sequences in proximity, and it facilitates signaling. The same result can be achieved with the leucine zipper chimeras, which mimic the placement of the engaged dimerization domains. This model is concordant with the differences observed in disposition of dimerization domain residues between the 1:1 and 2:1 complexes (31) and emphasizes the role of the dimerization domain in correct alignment of the GH receptor subunits, an element that is unimportant in the EPO receptor, which lacks such a defined domain.

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