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# Reversal of antigen-dependent signaling by two mutations in antibody/receptor chimera: implication of inverse agonism in cytokine receptor superfamily

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**Abstract**

Understanding the receptor activation mechanism is essential for the rational design of pharmacologically active ligand molecules. However, the activation mechanism of most cytokine receptors remains still unclear, and while agonism and antagonism have been described for ligand-mimetic peptides, there has been no report of inverse agonism that has been characterized for G protein-coupled receptors (GPCRs). To explore the activation mechanism of cytokine receptors, here we tried to investigate how agonism and antagonism could be altered by randomizing antibody variable region of an antibody/cytokine receptor chimera recognizing hen egg lysozyme (HEL) as an agonist. Based on our previous finding that the co-expression of $V_H$-gp130 and $V_L$-erythropoietin receptor (EpoR) chimeras transduced strict and efficient HEL-dependent cell growth signal, a $V_H$-gp130 library encoding four randomized CDR2 residues was retrovirally infected to IL-3-dependent Ba/F3 cells already transfected with $V_L$-EpoR. The selection without IL-3 resulted in a clonal expansion of the transduced cells, and interestingly some of which showed HEL dose-dependent growth suppression. Our results clearly indicate that agonism and antagonism of the antibody/cytokine receptor chimera can be readily switched by a subtle modification of the ligand binding domain as well as that of GPCRs, also implying the existence of inverse agonism in cytokine receptor superfamily.


*Keywords:* Inverse agonism; Erythropoietin receptor; gp130; Mutation; Antibody variable region; Chimeric receptor

## 1. Introduction

Many cellular events are triggered by transmembrane receptor (TMR) activation induced by the cognate ligands present in the extracellular milieu. Most normal TMRs known to date are switched off in the absence of their ligands, and ligand binding induces conformational change or oligomerization of the receptors, which triggers activation of the downstream signaling cascade. It is also known that the mutations in TMRs often lead to loss or gain of function that induces severe genetic disorders, indicating that receptors are important for the maintenance of homeostasis [1–5]. To cure the phenotypic abnormality or malignancy, many have tried to find agonists and/or antagonists to the specific receptors. On the other hand, antagonists with negative intrinsic activity called 'inverse agonists' were recently reported for G protein-coupled receptors (GPCRs), which constitute the largest family of TMRs [6,7]. Inverse agonists are especially advantageous in therapeutics, which intend to cure the diseases caused by a constitutively active receptor [7].

To establish an effective therapeutic protocol, the utmost importance is to know the nature and the activation mechanism of the target receptor. GPCRs have extensively been studied using approaches based on mutagenesis and chimeric receptors [8–10]. These studies not only provided insights into the receptor activation mechanism but also

*Abbreviations:* GPCR, G protein-coupled receptor; HEL, hen egg lysozyme; $V_H$, antibody variable region of heavy chain; $V_L$, antibody variable region of light chain; Fv, antibody variable region; EpoR, erythropoietin receptor; TMR, transmembrane receptor; gp130, glycoprotein 130; hL, human lysozyme; CDR, complementarity-determining region

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identified a wide variety of ligands that induce distinct signaling properties, leading to the concept of agonism, antagonism and inverse agonism [7,8,11,12].

In the case of cytokine receptors, which constitute another important family of TMRs, similar approaches have identified various types of ligands that behave as agonists or neutral antagonists. To date, these ligands include monoclonal antibodies [13,14], small peptides [15–17], and organic compounds [18–20]. Such molecules also contribute to the understanding of the activation mechanism of cytokine receptors. A marked example is a detailed comparison of the X-ray crystallographic structures of the extracellular portion of erythropoietin receptors (EpoR) that were unliganded or complexed with either Epo, agonist, or antagonist peptides, revealing that a small conformational change is critical for signal transduction [15,21–23].

These previous studies to find agonists or antagonists of cytokine receptors have been based on the robust screening of a diversified ligand library for a specific target receptor. On the other hand, few have been based on the reverse approach, where the cytokine receptor is randomized to screen those with agonistic or antagonistic properties to a specific ligand. In addition, while some constitutively active cytokine receptors have been reported [1,3,4], there has been neither the concept nor the experimental evidence of inverse agonism in cytokine receptors.

Previously, we have designed a series of hybrid antibody/receptor chimeras to confer the specificity to an exogenous ligand on cytokine receptors. We fused either $V_H$ or $V_L$ region of anti-hen egg lysozyme (HEL) antibody HyHEL-10 to extracellular D2 domain of erythropoietin receptor and transmembrane/cytoplasmic domains of either EpoR or gp130, resulting in four types of chimeric receptors. The co-expression of these chimeric receptors that reconstituted functional Fv induced HEL-dependent cell growth of factor-dependent hematopoietic and hybridoma cell lines [24–28]. By taking this system as a general model of ligand-inducible cytokine receptor, firstly we constructed a mutant chimeric receptor $V_H$-gp130 where four CDR2 residues, which were known to shift HEL specificity to that of human lysozyme (hL) [29], were mutated. However, when the mutant chimeric receptor was expressed in Ba/F3 already transfected with a $V_L$-EpoR (Ba/LE cells), cell growth was not induced with hL addition (M.K. et al, unpublished result). This result raised to us two questions: (i) how could agonism and antagonism be determined in cytokine receptors? and (ii) could inverse agonism exist as the nature of cytokine receptors as well as that of GPCRs? To address these issues, we randomized the above-mentioned four CDR2 residues critical for antigen binding to create a library of mutant $V_H$-gp130. Retroviral infection of the library into the Ba/LE cells followed by selections with or without HEL/hL resulted in the expansion of clones with functional chimeric receptors. Among several clones recovered, we found interesting clones whose growth was constitutively active but suppressed by the addition of antigen HEL, which was a typical property of inverse agonism. So we performed further characterization of these clones.

## 2. Materials and methods

### 2.1. Vector and library construction

The construction of Hg chain gene encoding HyHEL-10 $V_H$, a GSG linker, extracellular D2 domain of EpoR, and transmembrane/cytoplasmic domains of gp130 was described previously [25]. pMX [30] was digested with *Bpu*1102I and *Not*I, blunted with T4 DNA polymerase, and self-ligated to remove undesirable *Xho*I site, resulting in pMX-Xho. pMX-Hg [28] was digested with *Eco*RI and inserted into *Eco*RI-digested pMX-Xho to yield pMX-Hg-Xho. pMAMneo (Clontech, Palo Alto, CA) was digested with *Hin*dIII and *Xho*I, and a 1200 bp fragment was inserted into *Hin*dIII and *Xho*I-digested pMX-Hg-Xho to make pMX-L. In this step, $V_H$ gene was partially substituted with exogenous stuffer sequence derived from pMAMneo, to eliminate contamination of wild-type $V_H$ sequence in the library. The mutant $V_H$ library was generated by PCR with two primers VH-HindIII (5′-CCC-AAGCTTAGTCTCCATCCCCCAAACC-3′), and mVH-Xho (5′-CGCCTCGAGTACATGGGCTACGTCAGCNN-KNNKGGCNNKACCNNKTACAACCCCTCGCTG-3′), where the sequences corresponding to H53, H54, H56, and H58 were randomized (underlined). The amplified fragment was digested with *Hin*dIII and *Xho*I (double underlined), and ligated into *Hin*dIII and *Xho*I-digested pMX-L, followed by electroporation to *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA), resulting in approximately $1 \times 10^6$ colonies. All the colonies were mixed and expanded, from which the library vector named pMX-mHg was harvested with Plasmid Maxi Kit (Qiagen, Valencia, CA).

To isolate $V_H$ mutant genes from the selected cells, genomic DNA of the selected cells was prepared with Genomic Prep Cells and Tissue DNA Isolation Kit (Amersham-Pharmacia, Little Chalfont, UK), followed by PCR with two primers (pMX-BamHI: 5′-GATCCCAGTGTGG-TGGTAGGG-3′, EpoR N-term: 5′-GTCCAGGAGCACT-CCGGAACC-3′). The sequences for $V_H$ mutants were determined by Gene Rapid Seq 4x4 DNA sequencer (Amersham-Pharmacia). For genetically modified cell amplification assay, pMX-ScFvgIGFP (Kawahara M. et al., submitted) was digested with *Sac*II and *Sal*I, and inserted into *Sac*II- and *Sal*I-digested pMX-L to yield pMX-LIGFP. PCR-amplified mutant $V_H$ DNA from each clone was digested with *Xho*I and *Bsp*EI, and inserted into *Xho*I- and *Bsp*EI-digested pMX-LIGFP to obtain pMX-mHgIGFP.

To perform Open sandwich ELISA, mutant $V_H$ DNA from each clone was amplified by PCR with primers

pMX-BamHI and VHhelNotFor (5′-TTTGCGGCCGCC-GCCGAGACGGTGACGAGGGT-3′), digested with *Xho*I and *Not*I, and inserted to phagemid pluck2011 [31] digested with the same.

### 2.2. Cell culture

A murine IL-3-dependent pro-B cell line, Ba/F3 [32], was cultured in RPMI 1640 medium (Nissui Pharma, Tokyo, Japan) supplemented with 10% FBS (Iwaki, Tokyo, Japan) and 2 ng/ml of murine IL-3 (Genzyme/Techne, Cambridge, MA). A retroviral packaging cell line, Plat-E [33], was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1 μg/ml puromycin (Sigma, St. Louis, MO) and 10 μg/ml blasticidin (Kaken Pharmaceutical, Tokyo, Japan).

### 2.3. Retroviral transduction and selection

To make Ba/LE cells, Ba/F3 cells ($3 \times 10^6$ cells) were washed and resuspended with 500 μl Hanks' buffered saline (Nissui Pharma), and mixed with 10 μg pMEZ-LE [25]. The mixture was transferred to an electroporation cuvette, incubated for 10 min at room temperature, and then electroporated once with Electroporator II (Invitrogen, Groningen, The Netherlands) set at 250 μF and 660 V. After 10 min incubation at room temperature, cells were transferred to 10 ml medium in a Ø100 mm culture dish and incubated at 37 °C, under 5% $CO_2$ for 2 days, followed by a selection with 400 μg/ml zeocin (Invitrogen). The zeocin-resistant cells were cloned by limiting dilution, and a representative clone with LE expression was picked and named Ba/LE.

For mHg library infection, Plat-E cells were inoculated into two Ø100 mm dishes at $5 \times 10^5$ cells/ml in 10 ml DMEM containing 10% FBS, and cultured for 20 h. After dilution of 45 μl Fugene6 (Roche Diagnostics, Basel, Switzerland) in 500 μl of serum-free DMEM, the solution was added to 15 μg of the library vector dissolved in 5 μl of sterile water. After 15 min incubation at room temperature, the vector-Fugene6 mixture was added to the Plat-E cells. After 24 h incubation at 37 °C, the culture medium was refreshed with 10 ml DMEM containing 10% FBS, followed by 24 h incubation at 37 °C. After recovering the viral supernatant by centrifugation at $1000 \times g$ for 5 min at 20 °C, Ba/LE cells ($2 \times 10^6$ cells) were infected with 16 ml of the viral supernatant in the presence of 10 μg/ml polybrene (Sigma) and 1 ng/ml IL-3 in two Ø100 mm dishes. After 5 h incubation at 37 °C, 8 ml RPMI1640 containing 10% FBS was added to each dish to reduce the toxicity of polybrene. After incubation for a couple of days at 37 °C, the cells were washed three times with PBS, and inoculated into 96-well plates at $2 \times 10^5$ cells/ml (200 μl/well). Selection was performed in the medium containing no factor, 0.1, 1 or 10 μg/ml HEL (Seikagaku Corporation, Tokyo, Japan) or 0.1 or 1 μg/ml of human lysozyme (Sigma).

### 2.4. Western blotting

The cells ($10^6$ cells) were washed with PBS, lysed with 100 μl of lysis buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.5) and incubated on ice for 10 min. After centrifugation at $16000 \times g$ for 5 min, the supernatant was mixed with Laemmli's sample buffer and boiled. The lysate was resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). After the membrane was blocked with 1% BSA for phosphotyrosine detection or with 5% skimmed milk for others, the blot was probed with primary and HRP-conjugated secondary antibodies, and detection was performed using ECL system (Amersham-Pharmacia). The primary rabbit anti-mouse antibodies (anti-EpoR, anti-gp130, anti-STAT3, anti-STAT5, anti-ERK1/2) were from Santa Cruz Biotechnology (Santa Cruz, CA) except anti-phospho-ERK1/2 (Promega, Madison WI), and mouse anti-phosphotyrosine was from Transduction Labs (Lexington, KY). HRP-conjugated anti-rabbit IgG was from Biosource (Camarillo, CA), and HRP-conjugated anti-mouse IgG2b was from Zymed (South San Francisco, CA).

### 2.5. Luciferase reporter assay

A reporter plasmid pcfos-luc (375 ng) encoding firefly luciferase whose transcription is driven by c-fos promoter [34] and 18.75 ng of a control plasmid encoding *Renilla* luciferase, pRL-null (Promega), were diluted in 540 μl PBS containing 28 μl DEAE-dextran. $3.2 \times 10^6$ cells were washed once with PBS, resuspended in the DNA-DEAE-dextran mixture, and incubated for 30 min at room temperature. After centrifugation at $1000 \times g$ for 3 min, the cells were washed with PBS once, resuspended in RPMI1640 medium containing 10% FBS and 2 ng/ml IL-3, and incubated for 14 h. The cells were washed with PBS and depleted in RPMI1640 containing 10% FBS but without IL-3 for 5 h. The cells were stimulated with no factor, 10 μg/ml HEL or 1 μg/ml hL for 6 h. After harvesting the cells, the cell lysate was prepared and the activities of *Renilla* and firefly luciferases were detected with Dual Luciferase Reporter Assay System (Promega, Tokyo, Japan) and Luminescencer-JNR AB2100 (Atto, Tokyo, Japan).

### 2.6. Cell proliferation assay

The cells were washed with PBS and seeded in 24-well plates containing various concentrations of HEL. The initial cell concentration was adjusted to $4 \times 10^4$ cells/ml. Cell

number and viability were determined using a hemocytometer and the trypan blue exclusion assay.

### 2.7. Genetically modified cell amplification assay

Ba/F3 or Ba/LE cells were retrovirally infected with pMX-mHgIGFP as described. The approximate multiplicity of infection (MOI) ranged from 6.4 to 8.9% for Ba/F3 cells and from 34.3 to 47.9% for Ba/LE cells, as estimated from FACS analysis on Day 3 after infection. The infected cells were washed with PBS twice and seeded in 24-well plates containing either no factor, 1 μg/ml HEL or 2 ng/ml IL-3 eight days after infection. After selection, cells were harvested for Western blot analysis, and cell number and viability were determined using a hemocytometer and the trypan blue exclusion assay. FACS analysis was performed with FACSCalibur flow cytometer (Becton Dickinson, Lexington, KY) at 488 nm excitation and fluorescence detection at 530 ± 15 nm.

### 2.8. Affinity purification of chimeric receptors

The cells ($10^7$ cells) were washed with PBS containing 0.1% BSA (BSA-PBS) once, and incubated with biotinylated HEL for 90 min at 4 °C, followed by two washes with BSA-PBS. The cells were lysed with 1 ml lysis buffer, and incubated on ice for 10 min. After centrifugation at 16000 × *g* for 10 min, the supernatant was transferred to a microtube containing streptavidin-agarose (Sigma), and rotated for 60 min at 4 °C. The agarose was washed twice with wash buffer 1 (50 mM HEPES, 500 mM NaCl, 0.1% SDS, 0.2% Triton X-100, 5 mM EGTA, pH 7.5) and twice with wash buffer 2 (50 mM HEPES, 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 5 mM EGTA, pH 7.5), before boil with Laemmli's sample buffer. The supernatant was subjected to SDS-PAGE/Western blot analysis.

### 2.9. Immunoprecipitation

Cells were washed with PBS, lysed with 800 μl of lysis buffer, and incubated on ice for 10 min. After centrifugation, supernatant was mixed with anti-mouse STAT3 antibody (Santa Cruz) adsorbed on protein A-Sepharose (Amersham-Pharmacia) and rotated for 1 h at 4 °C. The sepharose was washed twice with wash buffer 1 and twice with wash buffer 2, and boiled with Laemmli's sample buffer. The supernatant was subjected to Western blot analysis.

### 2.10. Open sandwich ELISA

Preparation of $V_L$-immobilized plate and $V_H$-phage, and Open sandwich ELISA were essentially performed as described [31], except that $V_L$ fragment was immobilized through preadsorbed protein L (1 μg/ml, Actigen, Cambridge, UK).

## 3. Results

### 3.1. Construction of mutant $V_H$-chimeric receptor library

We first designed a library selection scheme to obtain functional chimeric receptors that confer growth advantage on Ba/F3 cells (Fig. 1). Randomized $V_H$ library tethered with a chimeric cytokine receptor was retrovirally transduced into an IL-3-dependent Ba/F3 cell clone transfected with $V_L$-receptor chimera, followed by selection with or without hen egg lysozyme or human lysozyme in the absence of IL-3. Since the Ba/F3 cell transfectant with a chimeric receptor combination of $V_H$-gp130 (Hg) and $V_L$-EpoR (LE) exhibited strict HEL-dependent cell growth [25,28], we chose a Ba/F3 cell transfectant with LE chain (Ba/LE) as a recipient.

Since a four amino acid mutation in the complementarity-determining region 2 of $V_H$ (HCDR2) of anti-HEL antibody HyHEL-10 had been found to confer increased affinity toward hL [29], a synthetic library of $V_H$ fragment was constructed by PCR using a mix primer to randomize the sequence corresponding to Y53, S54, S56, and Y58, which

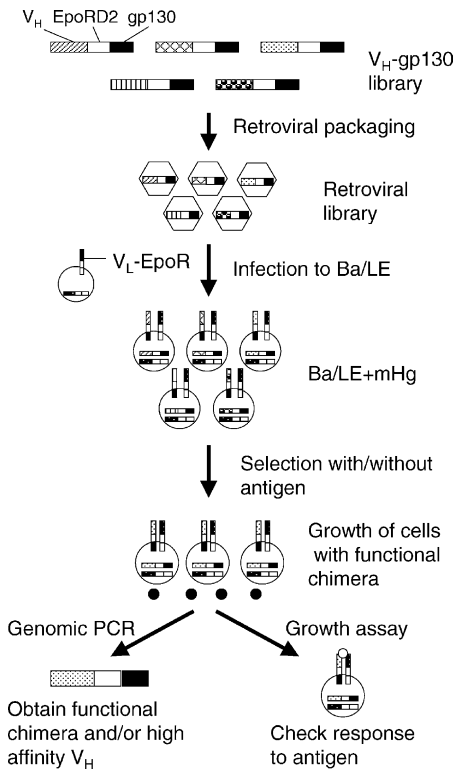


Fig. 1. The library selection scheme to obtain functional chimeric receptors. Randomized $V_H$ library tethered with a Gly-Ser-Gly linker, EpoR D2 domain and transmembrane/intracellular domains of gp130 is retrovirally infected into an IL-3-dependent Ba/F3 cells transfected with $V_L$-EpoR chimera (Ba/LE), followed by selection with or without a specific antigen in the absence of IL-3 to expand the cells with a functional chimeric receptor. Cell growth assay determines whether the cells could respond to the antigen. The functional $V_H$ sequences can be harvested and determined by genomic PCR and sequencing.

was ligated to the digested vector to obtain a mutant $V_H$-gp130 (mHg) library expression vector named pMX-mHg. Ba/LE cells were retrovirally transduced with pMX-mHg, followed by the selection in 96-well plates in a medium supplemented with no additional factors, hL or HEL. As a result, some colonies were found to grow without adding hL or HEL, although inclusion of HEL or hL resulted in slightly higher numbers of colonies (data not shown).

To characterize the nature of the colonies, the genomic DNA was extracted from each colony, and the sequence encoding the $V_H$ fragment was amplified by PCR to recover the integrated retroviral sequences (Table 1). Among 77 colonies analyzed, 19 gave a single sequence at the randomized positions, implying single copy integration of mHg in the genome of these clones, while the others showed degenerated sequences possibly due to multiple integrations of mHg chimeras with different sequences. Among single sequences, some apparently favored sequences (GSSF or GSRF) were repeatedly observed, while none had the wild-type sequence (YSSY). Most remarkable was the absence of Y53 in the selected clones. All encoded much smaller residues of G, S, N, and A at this position. Beside this position, S54 and S56 were relatively conserved among mutants. In addition, many clones shared Y58F or Y58L mutations, which were rather conservative mutations in size and shape of the side chain.

Table 1
Amino acid sequences for the selected Ba/LE + mHg clones

| Ligand | Amino acid sequence | Number of clones |
|---|---|---|
| None | GSSF | 1 |
| | GSRF | 1 |
| | Multi | 4 |
| 0.1 μg/ml hL | GSRF | 1 |
| | GSST | 1 |
| | SSRF | 1 |
| | Multi | 20 |
| 1 μg/ml hL | GSSF | 4 |
| | GSRF | 1 |
| | NNSL | 1 |
| | GGKF | 1 |
| | GNSV | 1 |
| | Multi | 14 |
| 0.1 μg/ml HEL | SNTL | 1 |
| | SSAF | 1 |
| | GSST | 1 |
| | ANRN | 1 |
| | Multi | 7 |
| 1 μg/ml HEL | SSAF | 1 |
| | Multi | 4 |
| 10 μg/ml HEL | ASDY | 1 |
| | Multi | 9 |
| Total | | 77 |

The amino acid sequences and their frequencies are indicated for each ligand concentration. Multi means that the sequences cannot be determined possibly due to multiple integrations of different mutants.

### 3.2. Some clones exhibited HEL-dependent growth suppression

To rapidly characterize the signal transduction efficiency of the selected clones, a luciferase reporter assay was performed. A previous report demonstrated that Ba/F3 transfectant with wild-type EpoR induced c-fos mRNA after Epo stimulation [35]. Therefore, the representative six clones were transiently transfected with a reporter plasmid encoding firefly luciferase driven by c-fos promoter, and a control plasmid encoding *Renilla* luciferase without promoter, and stimulated with/without HEL or hL. A clear HEL-dependent increase in specific luciferase activity was observed for Ba/LE cells that express Hg chain containing the wild-type $V_H$ sequence (Ba/LE + VHwt cells). On the other hand, all the selected clones exhibited elevated levels of luciferase activity before stimulation, and showed no apparent hL dependency. Among them, cells with GSSF and GSRF mutants (Ba/LE + GSSF and Ba/LE + GSRF cells, respectively) showed a marked HEL-dependent suppression of the luciferase activity (data not shown).

To confirm this rather unexpected finding, we examined activation patterns of other signaling molecules involved in EpoR- and/or gp130-derived signals. While both EpoR and gp130 activate Ras/MAPK pathway, the main STATs activated by EpoR and gp130 are STAT3 and STAT5, respectively [36,37]. Ba/LE + GSRF cells cultured with HEL were stimulated with various concentrations of HEL, followed by the detection of phosphorylation of STAT3, STAT5 and MAPK (ERK1/2) by Western blotting. While the level of STAT3 phosphorylation was considerably low in all HEL concentrations tested (Fig. 2A), the level of STAT5 phosphorylation was markedly increased by HEL deprivation, which was not observed in Ba/LE + VHwt cells (Fig. 2B). HEL also suppressed ERK1/2 phosphorylation, although the extent was less than that of STAT5 phosphorylation (Fig. 2C).

To further confirm HEL-dependent suppression of growth signals in Ba/LE + GSSF and Ba/LE + GSRF cells, these two clones were subjected to cell proliferation assay (Fig. 3A and B). Cells were washed and cultured in IL-3-free medium in the presence of various concentrations of HEL. Again, more than 1 μg/ml of HEL significantly suppressed cell growth of both clones, while 100 ng/ml HEL also suppressed growth of Ba/LE + GSRF cells to some extent.

### 3.3. Isolated clones exhibited high affinity binding to HEL

To examine whether the growth suppression was caused by the binding of HEL to the mutated receptors, cells with GSSF or GSRF mutant were incubated with biotinylated HEL, lysed and affinity-purified for the ligand-receptor complex with streptavidin-agarose beads.

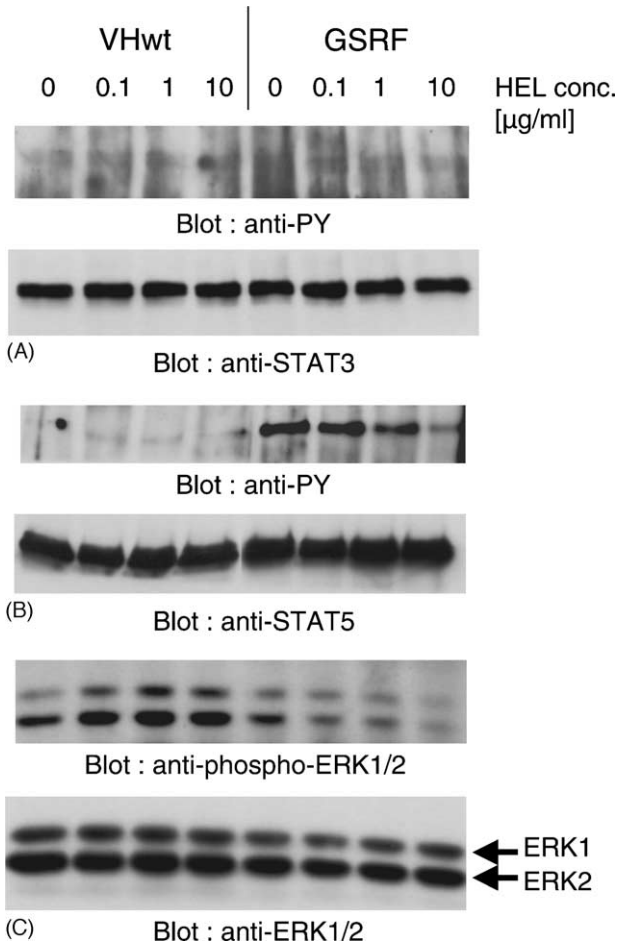


Fig. 2. Signal transduction to STAT3, STAT5, and ERK1/2. (A) Ba/LE + VHwt and Ba/LE + GSRF cells ($1.25 \times 10^7$ cells) cultured with HEL were washed with PBS twice and stimulated with or without HEL for 15 min at 37 °C, supplemented with ice-cold 1 mM $Na_3VO_4$ in PBS, pelleted and then lysed. The lysate was immunoprecipitated with anti-STAT3 antibody, followed by Western blot analysis with anti-phosphotyrosine (PY20) antibody or anti-STAT3 antibody for reprobing. (B) Tyrosine phosphorylation of STAT5 was detected by the same procedure as in (A), except the cell number used was $4.5 \times 10^6$ cells. (C) Tyrosine phosphorylation of ERK1/2 was detected by Western blot analysis using anti-phospho-ERK1/2 antibody.

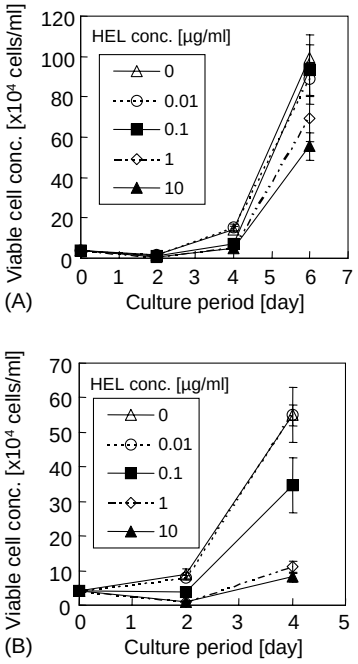


Fig. 3. HEL-dependent growth suppression of Ba/LE + GSSF and Ba/LE + GSRF cells. Cells ($4 \times 10^4$ cells/ml) were inoculated into 24-well plates at Day 0. Viable cell concentration in triplicates is plotted with average and 1 S.D. (A) Growth curve of Ba/LE + GSSF cells. (B) Growth curve of Ba/LE + GSRF cells.

According to the Western blots with anti-gp130 and anti-EpoR antibodies, both mHg and LE bands were detected in a biotinylated HEL dose-dependent manner, indicating specific binding of HEL to mHg and LE on the cell surface (Fig. 4).

To examine if each mutant $V_H$ domain per se can form HEL-dose dependent ternary complex of $V_H/V_L$/HEL, Open sandwich ELISA employing phage-displayed mutant $V_H$ and immobilized $V_L$ domains was performed. HyHEL-10 $V_L$ immobilized through protein L on microtiter plate wells was incubated with $V_H$-displaying phages together with various concentrations of HEL to test their relative $V_H/V_L$ interaction strengths. As shown in Fig. 5A, GSSF, GSRF mutants exhibited a little higher affinity to HEL than the wild type $V_H$, indicating preservation of the remarkable characteristic of HyHEL-10 that Fv is markedly stabilized by the antigen HEL. Open sandwich ELISA was further performed to compare the binding affinities of other mutants (Fig. 5B). SSAF mutant exhibited similar binding affinity to GSSF and GSRF mutants. On the other hand, GGKF showed a decreased, but still HEL-dependent $V_H/V_L$ interaction, while SNTL and NNSL mutants showed still lower interaction.

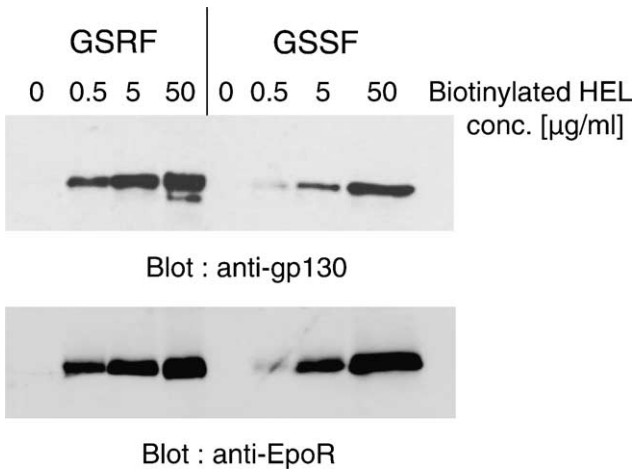


Fig. 4. HEL-affinity purification of mHg-HEL-LE complex on Ba/LE + mHg cell surface. Biotinylated HEL at the concentration indicated was added before cell lysis. Proteins complexed with biotinylated HEL were recovered from the lysate with streptavidin-agarose, followed by SDS-PAGE/Western blot analyses. The blotted proteins were probed with anti-gp130 antibody and reprobed with anti-EpoR antibody.

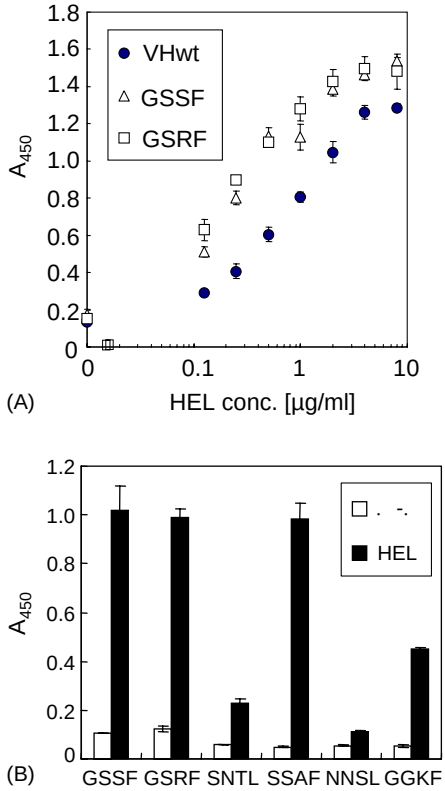


Fig. 5. Open sandwich assay showing mutant $V_H$-HEL-$V_L$ complex formation in vitro. (A) HEL-dependent $V_H$–$V_L$ interaction in wild-type $V_H$, GSSF and GSRF mutants. $V_L$ was immobilized through protein L onto the microtiter plate wells, followed by the addition of each $V_H$-displaying phage ($6 \times 10^{12}$ cfu/ml) in the presence of various concentrations of HEL. The amount of $V_H$-phage bound to immobilized $V_L$ was probed with HRP-labeled anti-M13 antibody. (B) Comparison of binding affinity among mutants. The mutant $V_H$-displaying phage ($3 \times 10^{12}$ cfu/ml) was added to the immobilized $V_L$ with or without 8 μg/ml HEL.

### 3.4. Background growth due to mHg-LE heterodimers

Based on these results, we reasoned two possibilities for the ligand-independent growth of Ba/LE + mHg cells. One was the heterodimerization of mHg and LE without any factors, where the preformed heterodimer was conformationally hyperactive and mediated strong signal transduction. The addition of HEL altered the receptor (Fv) conformation to a less active one, which led to apparent growth suppression. The other possibility was the homodimerization of mHg without any factors, where the homodimers were hyperactive and HEL addition induced less active mHg-LE heterodimers. To distinguish these two possibilities, and to re-examine the growth characteristics of Ba/LE + mHg clones, the six mHg expression vectors encoding each $V_H$ clone were reconstructed from the genomic PCR products, with an IRES-EGFP cassette to confirm the proper transduction of mHg to the cells. Ba/F3 and Ba/LE cells were retrovirally infected with each mHg expression vector, followed by the selection with or without HEL. When Ba/F3 cells were transduced, cells died immediately either in the presence or absence of HEL (data not shown). This result clearly ruled out the second possibility that mHg homodimers are hyperactive. On the other hand, all the transduced Ba/LE cells were viable either in the presence or absence of HEL, and all the viable cells strongly expressed EGFP according to FACS analysis (data not shown). Cell proliferation assay for the EGFP-amplified cells revealed that the cells with SSAF or GGKF mutant, which showed HEL binding in Open sandwich ELISA assay but no apparent HEL-dependency in luciferase reporter assay, also exhibited HEL-dependent growth suppression, although the effect was less than those of GSSF or GSRF mutant (Fig. 6A). While all the mutant Hg transduced cells selected without any factors exhibited background cell growth, this was not observed in the wild-type Hg transduced cells. To investigate whether or not the increased cell growth of the mutants was due to increased receptor expression levels, Western blot analysis was performed for the cells after selection (Fig. 6B). The expression levels of GSSF, SSAF and GGKF mHgs appeared to be almost comparable to or a slightly less than that of wild-type Hg, while GSRF mutant expressed slightly higher amounts than the wild type. It is notable that the SNTL and NNSL mutants expressed significantly higher amounts of two major bands shifted to higher molecular weights. The shift may be due to the *N*-linked glycosylation consensus sequences (Asn-X-Ser/Thr) created in these mutants. The relatively frequent appearance (3/11 different sets of mutations including GNSV mutant) of glycosylated forms implies that the increased receptor expression can enhance the growth signal intensity thereof. These results indicate that the $V_H$ mutation that caused inverse agonism induced the conformational change of preformed mHg-LE chimeric receptor, which exerted enhanced growth signal without addition of any factors (Fig. 7).

## 4. Discussion

Here we showed that as few as two mutations of the ligand-binding region of the antibody/cytokine receptor chimera turned the same ligand HEL from an agonist to an inverse agonist. The results clearly indicate that the agonistic behavior of the chimeric receptors can be readily switched by a subtle modification of ligand binding domain as well as that of GPCRs [9,10]. In addition, this would be the first demonstration of inverse agonism in the receptor assumed to have the same signal transduction mechanism to authentic cytokine receptors. Here we demonstrated inverse agonism in artificially induced EpoR-gp130 heterodimer that might not exist in nature. However, both EpoR and gp130 belong to type I cytokine receptor superfamily sharing the structural motifs and the signaling pathways such as JAK/STAT and Ras/MAPK. Furthermore, Hg + LE combination induced as efficient growth signal as homodimeric ones. Therefore, the results demonstrated here may reflect the nature of the wild-type cytokine

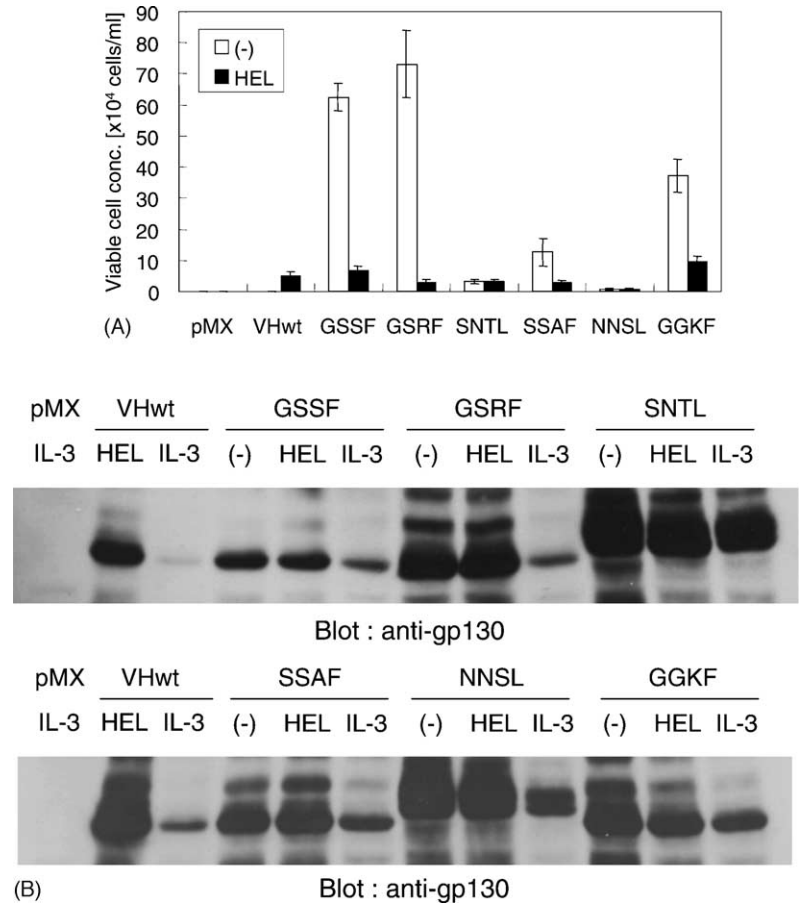


Fig. 6. Comparison of cell growth properties among Ba/LE + mHg cells with the genetically modified cell amplification assay. (A) Effect of HEL on cell growth of Ba/LE + mHgIGFP cells. Retrovirally infected cells ($4 \times 10^4$ cells/ml) were inoculated into 24-well plates at Day 0 and selected with or without 1 μg/ml HEL in the absence of IL-3. Viable cell concentration in triplicates on Day 5 is plotted with average and 1 S.D. pMX means Ba/LE cells transduced with pMX vector without any inserts as a negative control. (B) The mHg expression levels of the Ba/LE + mHg cells after selection. The cell lysates after selection with no factor (–), 1 μg/ml HEL or 1 ng/ml IL-3 were subjected to Western blot analysis using anti-gp130 antibody.

receptor system. In this study, only two amino acid substitutions in the antigen-binding region resulted in the constitutively active receptor, which is consistent with the previous reports that a small conformational difference affected receptor activation [15,21,38]. The result also implies that a limited point mutation of natural receptors can induce constitutive activation of the receptors, given that natural receptors are pre-associated [22,39,40]. In fact, some point mutations in constitutively activate receptors have been reported [1,3,4].

A number of agonists and antagonists recently identified for cytokine receptors could induce distinct conformations of the cognate receptor upon binding, partly due to their considerably different size and structure from those of natural cytokines [13,15–20,41]. Furthermore, alanine insertion mutagenesis in the juxtamembrane region of EpoR and gp130 revealed that orientation of the cytoplasmic domain is critical for signal transduction [38,42]. These studies clearly demonstrated that the receptor conformation determines whether a specific ligand could serve as an agonist or an antagonist. In this study, we showed the altered receptor conformation could turn the same ligand from an agonist to an inverse agonist, which is a marked expansion from the prior studies. It would be of interest whether the alanine insertion could further alter agonism/antagonism of the mutant chimeric receptors. In addition, we showed here that the constitutive growth activity induced by appropriate mutations was significantly higher than that of the wild-type receptor in response to HEL. Taken together, these results strongly indicate that cytokine receptors could also potentially adopt multiple activated conformational states as hypothesized in GPCRs [8]. In this sense, it is of quite interest how natural cytokine receptors could prevent a hyperactive conformational state in the absence of their cognate cytokines.

Among the analyzed mutants, the cells with GSSF, GSRF, SSAF or GGKF mutants exhibited HEL-dependent growth suppression with elevated levels of background growth. These mutants share the Y58F mutation and the G/S mutation at Y53. On the other hand, SNTL and NNSL mutants, which share Y58L mutation and S/N mutation at Y53, were constitutively active without HEL-dependency. These results suggest that the aromatic ring at position 58 is critical for HEL binding, which is consistent with the

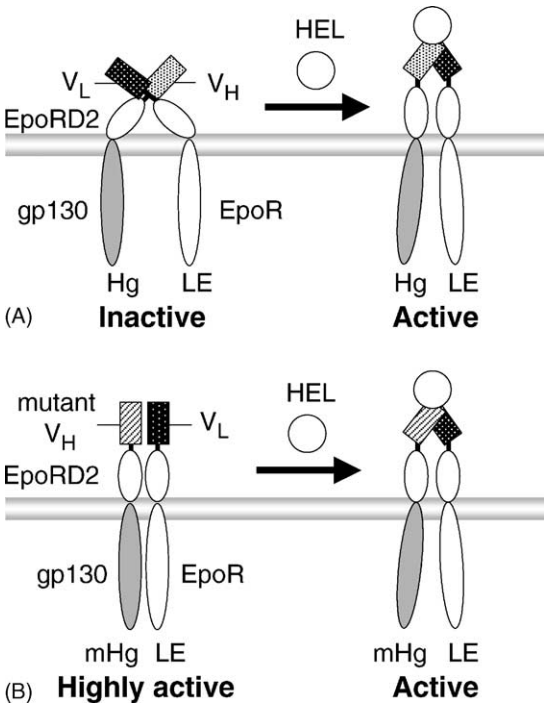


Fig. 7. Proposed mechanism of HEL-dependent Hg-LE heterodimer activation. (A) The activation mechanism for the wild-type Hg + LE chimeric receptor. (B) The activation mechanism for the mHg + LE chimeric receptors with the inverse agonistic property.

mutagenesis study of Fv fragments reported previously [43]. This hypothesis is further supported by the results that the cells with ASDY or SSRF mutant also exhibited HEL-dependent growth suppression (data not shown). The shared mutation at Y53 in all selected clones also implies the contribution of Y53 in preventing mHg from constitutive activation, although we cannot exclude the possibility of the additional contribution of the mutation at Y58. Y53G mutation induced significantly higher background growth than other mutants, indicating that flexibility and small size of Gly might accommodate the chimeric receptor with much favored conformation for signal transduction.

Here HEL suppressed the constitutive growth induced by subtle mutations in the chimeric cytokine receptor. Since many mutations related to disorders have been reported for receptor tyrosine kinases (RTKs) [2,44,45], it is possible that constitutively active RTKs also retain intrinsic property of inverse agonism. Unwinding of the nature and activation mechanisms of TMRs with diverse structural motifs will be scientifically attractive and provide great insight into the rational drug design.

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