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A Growth Signal with an Artificially Induced Erythropoietin Receptor-gp130 Cytoplasmic Domain Heterodimer[1]

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We report a strategy for generating efficient signal transduction with unnatural heterologous receptor combinations. As previously described [Ueda, H., Kawahara, M. *et al.* (2000) *J. Immunol. Methods* 241, 159–170], chimeric receptors composed of the V_H/V_L domains of anti-hen egg lysozyme antibody HyHEL-10 and N-terminally truncated erythropoietin receptor (EpoR) can be activated by lysozyme. When the cytoplasmic domains of these receptors were substituted with one derived from gp130, IL-3 dependent Ba/F3 cells expressing both V_H-gp130 and V_L-gp130 grew dose-dependently when given lysozyme without IL-3. However, cells expressing the heterologous pair of V_H-gp130 and V_L-EpoR also showed more efficient and stricter lysozyme-dependent proliferation in the absence of IL-3, indicating this combination is as an efficient and strict signal transducer as wild-type EpoR. The immunoprecipitation data indicated the existence of a preformed V_H-gp130 and V_L-EpoR heterodimer in the absence of lysozyme, suggesting the crucial role of a receptor conformational change in signal triggering as well as wild-type EpoR and gp130. Phosphorylation of JAK2, STAT3, and STAT5 was observed upon the addition of lysozyme, suggesting the activation of both EpoR- and gp130-derived signals.

Key words: antibody variable region, chimeric receptor, erythropoietin receptor, gp130, heterodimer.

Many cellular events are regulated through the association/dissociation of protein domains, and the methods for investigating and artificially controlling such events have been attracting considerable attention. Efforts have been made to induce the dimerization of signaling molecules by tethering specific binding domains to the signaling domain and administering a specific molecule to effect dimerization in the transfectant cells (*1–7*). Among these molecules, antigen antibody-based dimerizers should potentially have wider applications than ones comprising specific binding domains because there is an infinite number of possible antigen-antibody pairs. The current limitation of such approaches is that even single chain Fv (scFv) is relatively big (~25 kDa), and polyvalent antigen-mediated crosslinking of two binding domains tethered with signaling molecules such as growth factor receptors has not always led to a fully active conformation of the signaling domains *in vivo* (*8*)

We have been trying to establish a monomeric antigen-driven dimerizer of signaling molecules composed of separated antibody variable (V_H/V_L) domains each tethered with an N-terminally truncated erythropoietin receptor (EpoR) (*3*). While the V_H and V_L domains of anti-hen egg lysozyme (HEL) antibody HyHEL-10 exhibit low affinity to each other ($K_a < 10^5$ M^{-1}) on their own, the presence of an antigen drives the association of V_H and V_L ($K_a \sim 10^9$ M^{-1}) (*9*). In fact, when the chimeric Fv-EpoR was expressed in an IL-3 dependent pro-B cell line, Ba/F3, the receptor could transduce growth and apoptosis-suppression signals in response to the HEL concentration in IL-3 deficient medium. However, in this first system some cell growth was observed in the absence of the antigen, which might limit the usefulness of the system. Then a second system involving another IL-3 dependent cell line, 32D, was established, where a three-amino acid linker GSG was placed between the V region and EpoR domains of each chimeric receptor chain, V_H-EpoR (HE) or V_L-EpoR (LE). In this case, the transfectant 32D cells showed negligible growth in the absence of HEL and IL-3, and the inclusion of HEL in the media markedly stimulated cell growth. However, the viability of

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Abbreviations: EpoR, erythropoietin receptor; Fv, antibody variable domains, HEL, hen egg lysozyme; JAK, Janus kinase, STAT, signal transducers and activators of transcription, V_H, antibody variable heavy chain domain; V_L, antibody variable light chain domain

the transfectants at the early log phase decreased to ~60% even with the optimal HEL concentration of more than 200 ng/ml, and the resultant cell growth rate was not as good as that induced by cytokine stimulation.

As a way to ameliorate and expand current approaches, we here focus on the use of another subunit of the cytokine receptor family, gp130 (*10*). Among cytokines which play critical roles in the proliferation and differentiation of mammalian cells, there are at least six IL-6-type cytokines, *i.e.* IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin (CT)-1, each inducing pleiotropic biological responses (*11*). These cytokines all share gp130 as a common signal transducing subunit, which contributes to the functional redundancy of their actions. In the cytoplasmic domains of both gp130 and EpoR, box-1 and box-2 regions are involved in the binding of Janus kinases (JAKs), which play a pivotal role in signal transduction (*11, 12*). Gp130 activates JAK1, JAK2, and TYK2 (*11*), whereas EpoR only activates JAK2 (*13*). JAKs activate several signal transducers and activators of transcription (STATs), which translocate to the nucleus and bind to the specific enhancer sequence of target genes (*14*). So far, both gp130 and EpoR have been reported to activate three STATs, *i.e.* STAT1, STAT3, and STAT5 (*15, 16*), although the main STATs activated by gp130 and EpoR are STAT3 and STAT5, respectively (*17, 18*). Signal transducers other than JAKs and STATs, such as those in the Ras/MAP kinase pathway, are also activated by gp130 and EpoR (*19, 20*). These data suggest that the overall cytoplasmic domain structures and the range of activated signal transducers are distinct but similar between gp130 and EpoR. This leads to the question of whether or not induction of the EpoR-gp130 heterodimer, which has not been reported to exist in nature, could transduce a growth signal. To address this issue, we utilized our antibody/receptor chimera, which can selectively induce heterodimerization through antigen-dependent Fv formation.

MATERIALS AND METHODS

Plasmids—pME-HE encoding HyHEL-10 V_H, GSG linker, D2, and transmembrane and cytoplasmic domains of EpoR was described as pME-VHER(GSG) (*3*). pMEZ-LE was constructed from pME-VkER(GSG) (*3*) by replacing the G418 resistance and V_H genes of pME-HE with zeocin-resistance and V_L genes, respectively. Murine gp130 expression vector BCMGNeo-mgp130 (kindly provided by Dr. M. Hibi, Osaka University, Osaka) was digested with *Xho*I and then inserted into the *Xho*I site of pHSG396 to obtain pHSG396-gp. To remove undesirable restriction sites downstream of the gp130 sequence, the plasmid was digested with *Eco*RV and *Sal*I, blunted with T4 polymerase, and then self-ligated (pHSG396-gp-2). pHSG396-gp-2 was digested with *Hin*dIII and *Bpu*1102 I to remove the gp130 extracellular sequence, where a synthesized linker made of two oligonucleotides (5′-AGCTTGATATCGCCATAGTCGTGCCTGTTTGC-3′, 5′-TAAGCAAACAGGCACGACTATGGCGATATCA-3′) was inserted to place an *Eco*RV site (underlined) at the N-terminus of the gp130 transmembrane sequence, designated as pHSG396-intragp130. pME-HE and pMEZ-LE were digested with *Eco*RV and *Xba*I to remove the EpoR intracellular domain, and the *Eco*RV-*Xba*I fragment of pHSG396-intragp130 (transmembrane and intracellular domains of gp130) was inserted to obtain pME-Hg and pMEZ-Lg, which are expression vectors for Hg and Lg, respectively.

Cell Culture—A murine IL-3–dependent pro-B cell line, Ba/F3 (*21*), was cultured in RPMI 1640 medium (Nissui, Tokyo) supplemented with 10% FBS (Iwaki, Tokyo) and 2 ng/ml murine IL-3 (Genzyme, Cambridge, MA), unless otherwise indicated.

Transfection and Selection—Ba/F3 cells (3×10^6 cells) were washed and resuspended with 500 μl Hanks' buffered saline (Nissui), and then mixed with 10 μg each of pME-Hg and pMEZ-Lg. The mixture was transferred to a 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 ϕ100 mm culture dish and incubated at 37°C, under 5% CO_2 for 2 days, followed by selection with 800 μg/ml G418 (Sigma, St. Louis, MO) and 400 μg/ml zeocin (Invitrogen). The antibiotic-resistant cells were further selected in the IL-3 deficient medium containing 10 μg/ml HEL (Seikagaku, Tokyo) and cloned by limiting dilution. A representative clone was named Ba/Hg+Lg and used for further study. Likewise, Ba/F3 cells were transfected with pME-Hg and pMEZ-LE to establish Ba/Hg+LE cells.

Western Blotting—10^6 cells were washed with PBS, lysed with 100 μl 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 then incubated on ice for 10 min. After centrifugation, the supernatant was mixed with Laemmli sample buffer and boiled for SDS-PAGE/Western blot analysis. The blot was serially probed with primary and HRP-conjugated secondary antibodies, and detected with an ECL system (Amersham-Pharmacia, Buckinghamshire, UK). The primary rabbit anti-mouse antibodies (anti-gp130, anti-EpoR, anti-STAT3, and anti-STAT5b) were from Santa Cruz Biotechnology (Santa Cruz, CA), except anti-JAK2 (Upstate Biotechnology, Lake Placid, NY), 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).

Cell Proliferation Assay—Cells were washed three times with PBS and seeded into 24-well plates with various concentrations of HEL. Cell concentration and viability were measured with a hemocytometer by means of the trypan blue exclusion assay.

Affinity Purification of Chimeric Receptors—For the purification of chimeric receptors from a lysate, 10^7 cells were washed with PBS containing 0.1% BSA (BSA-PBS) three times, lysed with 1 ml lysis buffer, and then incubated on ice for 10 min. After centrifugation, the supernatant was mixed with biotinylated HEL for 90 min at 4°C, transferred to a streptavidin-agarose (Sigma)–containing tube, and then 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 then 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 boiling with Laemmli sample buffer. The superna-

tant was subjected to SDS-PAGE/Western blot analysis.

For cell surface labeling, 10^7 cells were washed with BSA-PBS three times and incubated with biotinylated HEL for 90 min at 4°C, followed by three washes with BSA-PBS and cell lysis. The same procedure as above was performed after the addition of streptavidin-agarose to the cell lysate.

For the immunoprecipitation of chimeric receptors with antibodies, the same procedure as above was performed except that 500 μl lysate from 5×10^6 cells and antibody-bound protein A–Sepharose (Amersham-Pharmacia) were mixed for 60 min.

RESULTS

HEL-Responsive Growth of the Ba/F3 Transfectant Expressing Chimeric Receptors Having the gp130 Cytoplasmic Domain—To determine whether or not the exchange of our Fv-EpoR cytoplasmic domain for that of gp130 yields a functional chimeric receptor, Ba/F3 cells were cotransfected with the expression vectors for V_H-GSG-EpoR-gp130 (Hg) and V_L-GSG-EpoR-gp130 (Lg) chimeras, and then selected in normal medium containing IL-3 and two antibiotics. When the selected cells were cultured in the medium containing HEL but without IL-3, a part of the cells survived and started to grow These cells were then cloned, a representative clone being named Ba/Hg+Lg. When the expression of both chimeric receptor chains in Ba/Hg+Lg cells was confirmed by Western blotting, similar amounts of Hg and Lg proteins were detected (Fig 1C, Hg+Lg).

To see whether or not the growth of Ba/Hg+Lg cells could be stimulated by HEL addition in IL-3 deficient medium, cells were washed and cultured in various concentrations of HEL for 6 days, the time courses of viable cell concentration and viability being plotted (Fig. 2, A and B). As expected, the cells proliferated in a HEL concentration–de-

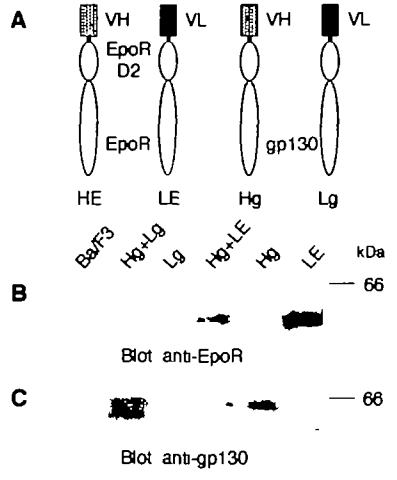

Fig 1 **Expression of chimeric receptors in Ba/F3 transfectants.** (A) Schematic representation of chimeric receptors Hg and Lg were generated by replacing the intracellular domains of HE and LE with that of gp130 The co-expression of two chimeric receptor chains in Ba/Hg+Lg and Ba/Hg+LE clones was confirmed by Western blotting with (B) anti–EpoR carboxy terminus antibodies and (C) anti-gp130 carboxy terminus antibodies. The respective chimeric receptor bands for Ba/Hg+Lg and Ba/Hg+LE cells are compared with that for Ba/F3 cells transfected with either pME-Hg (Hg), pMEZ-Lg (Lg), or pMEZ-LE (LE), respectively

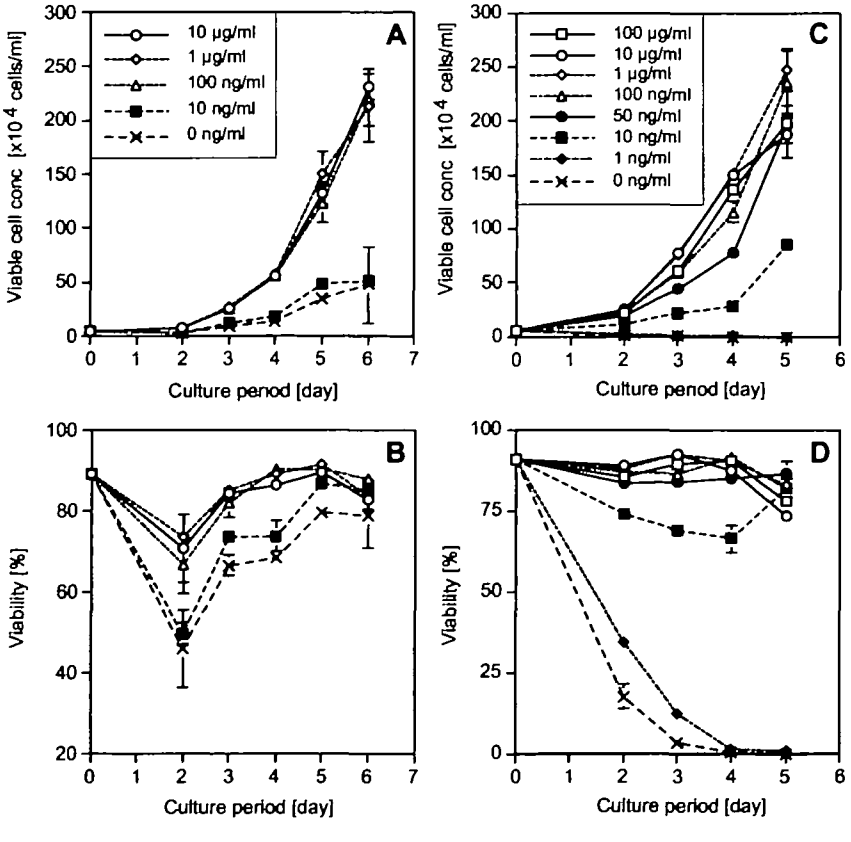

Fig 2 **Growth and viability curves of Ba/Hg+Lg and Ba/Hg+LE cells.** Cells (5×10^4 cells/ml) were inoculated into 24-well plates at day 0 The viable cell concentration, triplicate determinations is plotted with average and 1 SD (A) HEL concentration-dependent growth of Ba/Hg+Lg cells. (B) Viability of Ba/Hg+Lg cells, where the symbols are the same as in (A). (C) HEL concentration-dependent growth of Ba/Hg+LE cells. (D) Viability of Ba/Hg+LE cells, where the symbols are the same as in (C).

pendent manner, and the growth rate became maximum when more than 100 ng/ml HEL was added. However, some basal growth was observed without HEL addition, which was similar to the results previously obtained for Fv-EpoR transfected Ba/F3 cells.

Establishment of Ba/F3 Transfectants Expressing a Chimeric Receptor with Heterodimeric EpoR/gp130 Cytoplasmic Regions—We then speculated on the possibility that the induction of EpoR and gp130 cytoplasmic domain heterodimerization might trigger a cell growth signal as well as the respective homodimerizations. To see whether or not heterogeneous receptor chains having gp130 and EpoR cytoplasmic regions could transduce a growth signal, Ba/F3 cells were cotransfected with the expression vectors for V_H-GSG-EpoR-gp130 (Hg) and V_L-GSG-EpoR (LE), and selected as before. To our surprise, a part of the antibiotic resistant cells could survive and proliferate when cultured in HEL^+ $IL\text{-}3^-$ medium The cells were cloned and named Ba/Hg+LE. Western blot analysis showed the simultaneous expression of Hg and LE in Ba/Hg+LE cells. This was confirmed by the comigration of the corresponding bands for Ba/Hg and Ba/LE, which are Ba/F3 transfectants expressing Hg or LE, respectively (Fig 1, B and C).

HEL-Dependent Cell Growth via Hg-LE Heterodimers—Ba/Hg+LE cells were washed and cultured in the IL-3 deficient medium containing various concentrations of HEL for 5 days, and then the time courses of viable cell concentration and viability were plotted (Fig. 2, C and D). As expected, HEL induced cell proliferation in a dose-dependent manner, while cells died within 3 days without HEL addition. When 50 to 1,000 ng/ml HEL was added, the final cell concentration of more than 2×10^6 cells/ml, as well as viability of >85% before overgrowth, was attained. The values obtained were almost the same as those obtained earlier for the Epo response of Ba/F3 cells transfected with wild-type EpoR (*3*). The lowest necessary HEL concentration to maintain the growth was 10 ng/ml, which is comparable to the value previously observed on Open Sandwich ELISA (*9*), although 1 ng/ml HEL showed some anti-apoptotic effect When cell proliferation assays for Ba/Hg, Ba/LE, and Ba/Lg cells (5×10^4 cells/ml at day 0) were performed, none of them showed a growth response to HEL, resulting in complete cell death at most within 5 days (data not shown). These results indicate that the Hg-LE heterodimer transduces efficient cell growth and anti-apoptotic signals in response to HEL. To explore the unexpectedly good HEL-dependent growth response of Ba/Hg+LE cells, further characterization of the cells was performed.

Formation of a HEL-Hg-LE Complex—To ensure the ternary complex formation of HEL, Hg, and LE during the cell culture with HEL, the cell lysate was mixed with or without biotinylated HEL, followed by affinity purification of the ligand-receptor complex with streptavidin-agarose beads. Western blot analysis showed that both Hg and LE were detected in the samples to which biotinylated HEL was added (Fig. 3, A and B) The biotinylated HEL-dependent bands for Hg and LE were even observed when the

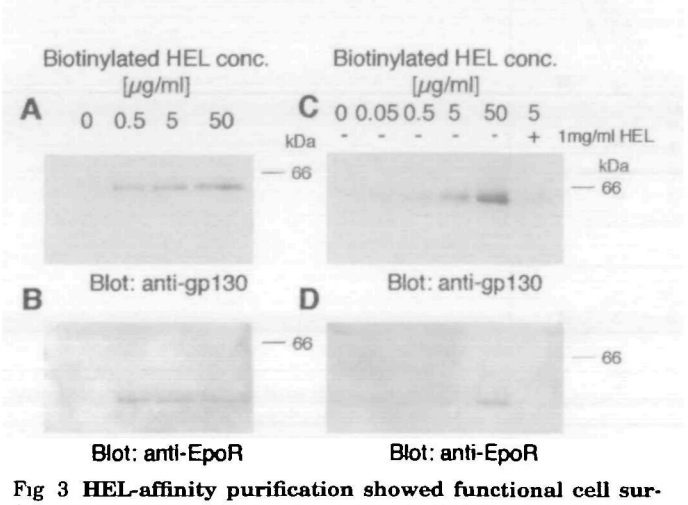

Fig 3 **HEL-affinity purification showed functional cell surface Hg-LE-HEL complex formation in Ba/Hg+LE cells.** Biotinylated HEL at the concentrations indicated was added either after (A and B) or before (C and D) cell lysis. Proteins complexed with biotinylated HEL were recovered from the lysates with streptavidin-agarose The blotted proteins were probed with anti-gp130 antibodies (A and C) and reprobed with anti-EpoR antibodies (B and D).

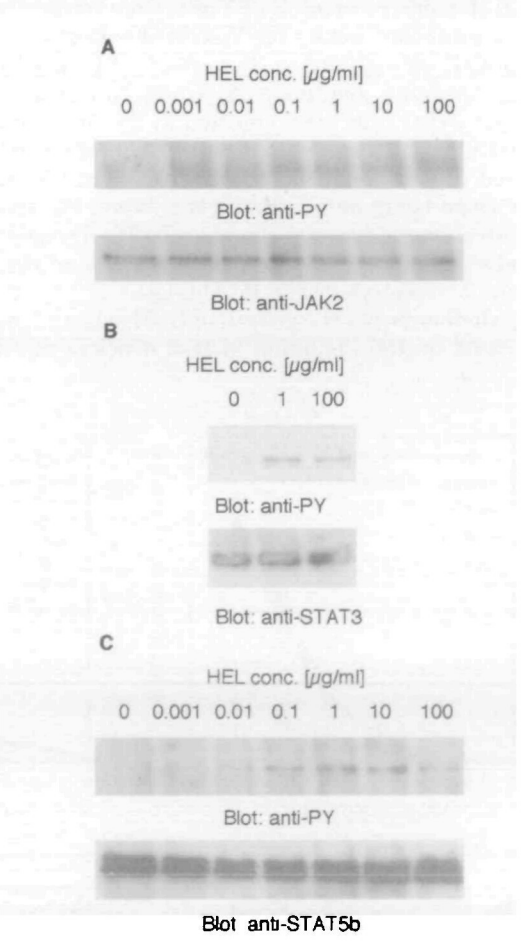

Fig 4 **Tyrosine phosphorylation of JAK2, STAT3, and STAT5b.** (A) Exponentially growing Ba/Hg+LE cells (7.5×10^6 cells) were washed three times with PBS and then starved in the depletion media (RPMI1640, 10% FBS) for 21 h at 37°C Cells were stimulated with HEL for 15 min at 37°C, supplemented with ice-cold 1 mM Na_3VO_4 in PBS, pelleted and then lysed The lysate was immunoprecipitated with anti-JAK2 antibodies, followed by Western blot analysis with anti-phosphotyrosine (PY20) antibodies or anti-JAK2 antibodies for reprobing. In (B) and (C), tyrosine phosphorylation of STAT3 and STAT5b was detected by the same procedure as in (A). In both cases, the cell number used was 10^7 cells, but the depletion times were 16 and 21 h for STAT3 and STAT5b, respectively

cells were mixed with biotinylated HEL and extensively washed prior to cell lysis (Fig. 3, C and D). The addition of excess HEL abolished both the Hg and LE bands. In our previous experiment, either V_H or V_L alone exhibited far lower affinity ($K_a \leq 10^7$/M) to HEL (*9*) In addition, the rate of dissociation of the VH-VL-HEL complex of 2.73×10^{-6}/s (~0 1/h) is much lower than that of VH-HEL (2.42×10^{-4}/s) or VL-HEL (8.9×10^{-3}/s) (HU, unpublished result). So it is highly likely that only the Hg-LE-HEL complex could survive a long immunoprecipitation and washing procedure. These results indicate the specific binding of HEL to Hg and LE on the cell surface to form a HEL-Hg-LE complex during the cell culture in HEL$^+$ medium.

HEL Induced Tyrosine Phosphorylation of JAK and STATs—JAK2 is known to be commonly activated by wild-type EpoR and gp130. Therefore, we investigated whether or not JAK2 is phosphorylated when the gp130-EpoR cytoplasmic domain heterodimer is induced by HEL. Cells were starved in IL-3$^-$ HEL$^-$ medium, and then stimulated with 1 ng/ml to 100 μg/ml HEL for 15 min and immunoprecipitated with anti-JAK2 antibodies, followed by Western blot with anti-phosphotyrosine antibodies (Fig. 4A). While the amount of immunoprecipitated JAK2 was almost the same in each lane, increased band densities for phosphorylated JAK2 were detected in the HEL$^+$ lanes, indicating HEL induced tyrosine phosphorylation

STAT3 and STAT5 have been reported to be the main STATs activated by gp130 and EpoR, respectively, which are recruited to the receptors and tyrosine-phosphorylated by JAKs including JAK2 to be activated (*17, 18*). Therefore, we also investigated whether or not STAT3 and STAT5b are phosphorylated when stimulated with HEL. While the amount of immunoprecipitated STAT was almost the same in each lane, increased band densities for phosphorylated STATs were detected in the HEL$^+$ lanes, indicating HEL induced tyrosine phosphorylation of both STAT3 and STAT5b (Fig 4, B and C) These results imply that both Hg and LE were activated, and both gp130 and EpoR signals were transduced in response to HEL.

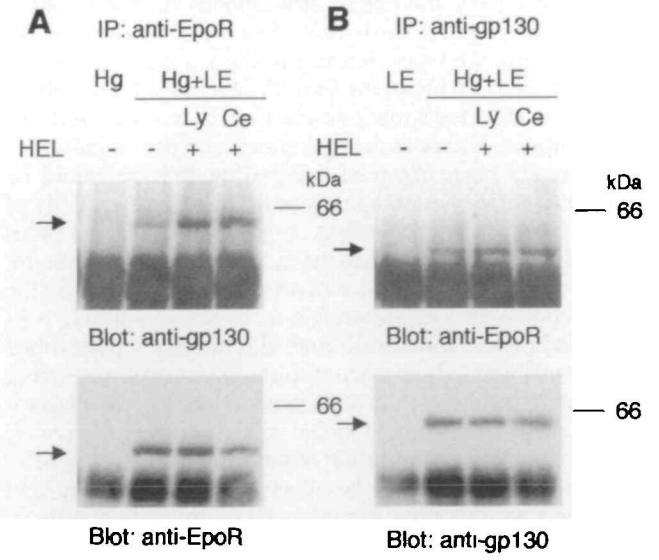

Fig. 5. **Immunoprecipitation revealed Hg-LE preformed dimers.** Ba/Hg+LE cells (5×10^6 cells) cultured in the medium containing IL-3, G418, and zeocin but no HEL were washed twice with PBS, and 100 μg/ml HEL was added either after (Ly) or before (Ce) cell lysis. The lysate was immunoprecipitated with anti-EpoR (A) or anti-gp130 antibodies (B), and then probed with anti-gp130 or anti-EpoR antibodies, respectively. As controls, Ba/Hg and Ba/LE cells (10^7 cells each) were also used.

Preformed Dimer of Hg and LE—To obtain an idea about the receptor activation mechanism, cells were incubated with or without HEL, lysed and then immunoprecipitated with anti-EpoR antibodies, followed by Western blotting with anti-gp130 antibodies. Interestingly, a Hg band was clearly detected even without HEL addition and the density of the Hg band seemed to slightly increase with HEL addition (Fig. 5A). To exclude the possibility of nonspecific recognition of Hg by anti-EpoR antibodies, a Ba/Hg cell lysate was prepared and immunoprecipitated with anti-EpoR. No Hg band was observed on Western blotting (Fig. 5A), indicating specific recognition of LE by anti-EpoR. The same result was obtained in the case of immunoprecipitation with anti-gp130 and blotting with anti-EpoR (Fig. 5B). The LE band was clearly detected even without HEL addition. Again, the possibility of nonspecific binding of anti-gp130 to LE was excluded by the immunoprecipitation with a Ba/LE cell lysate (Fig. 5B). These results indicate that most of the Hg-LE complex exists as a preformed dimer and that a HEL-induced conformational change is necessary for the cell growth signal transduction.

DISCUSSION

With the intent of widening the cell specificity to be controlled, we changed the cytoplasmic domain of our antibody Fv/EpoR chimera to that of gp130. While this strategy worked well in the case of IL-3 dependent Ba/F3 cells and an IL-6 dependent 7TD1 hybridoma (*22*), it was unexpected that the heterodimers of EpoR and gp130 cytoplasmic domains exert similar and even better antigen dose-dependent growth signal transduction in Ba/F3 cells than the corresponding homodimers The viability with HEL was maintained at as high as that with wild-type EpoR signaling, while the cell growth was strictly HEL-dependent. Gp130 is an efficient signal transducing subunit for the IL-6 family of cytokines in the cases of the LIFR-gp130 and OSMR-gp130 heterodimers as well as the gp130 homodimer. In the case of EpoR, Epo-induced heterooligomers of EpoR and IL-3Rβc (*23, 24*), and stem cell factor-induced heterooligomers of c-Kit and EpoR (*25, 26*) have been reported. However, heterologous receptor combinations between EpoR and IL-6 family receptors, such as the EpoR-gp130 heterodimer, have not been reported. Therefore, this study is the first to demonstrate that an unnatural EpoR-gp130 cytoplasmic domain heterodimer can transduce an efficient growth signal. Although signal cross-talk between distinct receptor systems can in principle be discovered by adding the respective agonists to the receptor-expressing cells, our inducible heterodimer system has proven to be an alternative means of discovering unprecedented cross-talk between different families of signaling molecules, which can also lead to its application as an improved signal transducer.

Recently, X-ray crystallographic and other analyses suggested a complicated activation mechanism for EpoR. According to the reports, EpoR exists as a preformed dimer with its two cytoplasmic domains apart from each other

(*27–29*). Epo addition induces a conformational change bringing the two EpoR cytoplasmic domains into close proximity, which triggers signal transduction. On the other hand, comparison between agonist (EMP1)-EpoR and antagonist (EMP33)-EpoR complexes revealed that small differences in the EpoR extracellular domain orientation determine whether these peptide ligands act as agonists or antagonists (*30, 31*). Moreover, a recent report showed that gp130 is not activated by dimerization with the addition of a single antibody alone, the addition of a second antibody Fab fragment being required to induce an active conformation of the gp130 dimer for signal transduction (*32*). Recent reports have also suggested that other receptors such as receptors for epidermal growth factor and IL-2 exist as preformed dimers (*33–35*). Like these reports, our results also suggest that the receptor conformation is critical for signal transduction. In Ba/Hg+LE cells, proliferation was stimulated only when HEL was added to the culture medium, even though the Hg-LE heterodimer had been formed prior to HEL addition, as suggested by the immunoprecipitation. This strongly suggests that HEL induced a conformational change of the Hg-LE dimer as the activation mechanism (Fig. 6). In contrast, Ba/Hg+Lg cells showed some proliferation before HEL addition, indicating that Hg-Lg dimers were already in some reduced active state. The addition of HEL possibly leads to a more active receptor through further conformational changes. These results suggest that the conformation is critical for the EpoR-gp130 cytoplasmic domain heterodimer as well as the gp130 homodimer, and that small conformational differences have an effect on signal transduction, which may further potentiate the value of the Hg+LE combination.

It could be a demerit of our system that some artificial effect other than the simple approximation of cytoplasmic domains could also be induced on tethering of the cytoplasmic domains with the exogenous extracellular portions. For example, a preformed dimer may elicit some background signal without antigen addition, as seen in the case of Hg+Lg Nevertheless, we believe that the apparent similarity of the activation mechanism of our chimeric receptor to that of wild-type cytokine receptors has some advantage over conventional dimerization inducers such as an FKBP-based system (*1*), given the receptor activation mechanism is not so simple. Our system also utilizes a non-natural agonist, which is also advantageous compared to other natural receptor-based heterodimerizers, where endogenous signaling or cross-talks might give considerable background.

In our chimeric receptor system, STAT3 and STAT5b were phosphorylated through HEL stimulation, suggesting that JAKs associated with gp130 and EpoR were also activated since gp130 and EpoR mainly activate STAT3 and STAT5, respectively. Recent reports have shown that gp130 activates three STATs (*i.e.* STAT1, STAT3, and STAT5), but the activation mechanism of STAT5 is different from that of the others. STAT1 and STAT3 bind to phosphotyrosine on the receptor, and are phosphorylated by JAKs, whereas STAT5 is directly associated with JAKs and phosphorylated (*15*). EpoR also activates these three STATs, although the activation mechanism of STAT1 and STAT3 has not been elucidated (*16*) Whether or not the activated STATs are derived from either receptor-recruited or JAK-associated stores is an interesting question with regards to our system, where different STAT recruitment patterns are mixed together. The activation pattern of signal transducers other than JAK and STATs is also of interest since specific signal transducers may be activated by the artificial heterodimer. The higher cell viability than that observed with our previous chimeric receptor system might imply efficient anti-apoptotic signal transduction through systems such as Bcl-x_L and Akt (*36, 37*). Finally, the application of the Hg-LE dimer to other cell types might induce an unexpected response, leading to the discovery of as-yet-unidentified signals such as stem cell renewal and/or lineage-specific differentiation ones.

There are almost infinite combinations of antigen–antibody pairs. This means that various antibody/receptor chimeras responsive to a variety of antigens could in principle be obtained. Although it will be necessary to find Fv fragments in which V_H and V_L can be driven together by an antigen, the approach might be general within these limitations, and allow the use of any monomeric antigen as a ligand to direct cell growth. Such Fvs could be identified in advance using the Open Sandwich assay, which detects the stabilization of Fv by an antigen (*9, 38*). Indeed, a HyHEL-10 variant that recognizes human lysozyme (*39*) and another suitable Fv recognizing a much smaller hapten (*40*) have already been obtained, suggesting that it should be possible to generalize this approach. The strict specificity of antibody–antigen interactions, especially compared with the highly redundant cytokine-cytokine receptor system, should enable very precise control of the growth of specific cells of choice. This might enable us to obtain suitable candidate pairs for industrial and therapeutic applications such as efficient cell growth stimulation/control employing inexpensive antigens in protein production (*41*), or selective expansion of genetically modified cells *in vitro* or *in vivo* (*1, 2, 7, 42, 43*). Since stimulation of both EpoR (*44*) and gp130 (*45*) has been reported to be effective for maintaining and expanding multipotent hematopoietic progenitor cells, a Hg+LE heterodimer containing the two signaling domains may be ideal for specifically and reversibly expanding genetically modified hematopoietic cell populations.

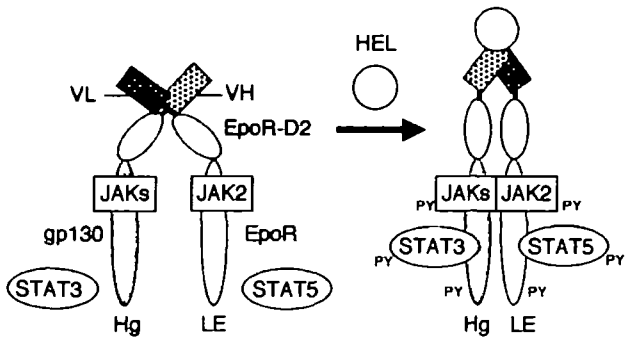

Fig. 6 **Proposed mechanism of HEL-induced Hg-LE heterodimer activation.** The Hg-LE interaction may be mediated by the extracellular domains rather than the intracellular ones, since there seems to be no interaction between wild type EpoR and gp130.

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