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Mimicry of Erythropoietin and Interleukin-6 Signalling by an Antibody/Cytokine Receptor Chimera in Murine Myeloid 32D Cells

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We have previously designed antibody-cytokine receptor chimeras that could respond to a cognate antigen. While these chimeric receptors were functional, it has not been investigated exactly how they mimic signal transduction through corresponding wild-type receptors. In this study, we compared the growth properties and the phosphorylation status of intracellular signal transducers between the erythropoietin receptor (EpoR)- or gp130-based chimeric receptors and wild-type EpoR or EpoR-gp130 chimera, respectively. Expression plasmids, encoding V_H or V_L region of anti-hen egg lysozyme (HEL) antibody HyHEL-10 tethered to a pair of extracellular D2 domain of EpoR and transmembrane/cytoplasmic domains of either EpoR or gp130, were constructed, and pairs of chimeric receptor combinations (V_H-EpoR and V_L-EpoR, V_H-gp130 and V_L-gp130, V_H-EpoR and V_L-gp130, V_H-gp130 and V_L-EpoR) were expressed in an IL-3-dependent myeloid cell line, 32D. Growth assay revealed that the transfectants all grew in a HEL-dependent manner. As for phosphorylation of Stat3, Stat5, ERK and Akt, the chimeric receptors showed similar activation pattern of signalling molecules with wild-type receptors, although the chimeric receptors showed ligand-independency and a little lower maximal phosphorylation than the corresponding wild-type receptors. The results demonstrate that antibody-receptor chimeras could substantially mimic wild-type receptors.

Key words: antibody, chimeric receptor, erythropoietin receptor, gp130, haematopoietic cell.

Abbreviations: Epo, erythropoietin; EpoR, erythropoietin receptor; HEL, hen egg lysozyme; Stat, signal transducers and activators of transcriptions; V_H, antibody variable domain of heavy chain; V_L, antibody variable domain of light chain.

Cytokines play a pivotal role in regulating cellular functions, such as growth, differentiation and death. Cytokines trigger signal transduction by binding to their cognate receptors on the cell membrane. Type I cytokine receptors share common motifs of a conserved four-cysteine motif in the extracellular domain, a WSXWS motif in the membrane-proximal portion of the extracellular domain, immunoglobulin-like or fibronectin type III-like domains, a single transmembrane domain and no kinase-like domain in the intracellular domain (*1*). Recently, X-ray crystallographic analyses (*2*, *3*) and fluorescence resonance energy transfer (FRET) assays (*4–6*), as well as dihydrofolate reductase (DHFR) (*7*) and β-galactosidase complementation assays (*8*), have suggested that type I cytokine receptors are activated, not only by ligand-induced dimerization, but also by conformational change.

In order to analyse and mimic the functions of type I cytokine receptors, many investigators have designed various chimeric receptors, where the extracellular domain was changed to that of other receptors (*9–15*). These chimeric receptors could transduce a growth signal in response to a number of ligands in addition to endogenous cytokines. Truncation and mutagenesis approaches have revealed the critical portion and residues in the receptors of interest. In addition to these analytical approaches, we have utilized antibody variable domains to engineer chimeric cytokine receptors, which could respond to their cognate antigens. For example, we fused either the V_H or V_L region of the anti-hen egg lysozyme (HEL) antibody HyHEL-10 to the extracellular D2 domain of erythropoietin receptor (EpoR) and transmembrane/cytoplasmic domains of either EpoR or gp130, resulting in the creation of four chimeric receptor chains. Co-expression of these chimeric chains that reconstituted functional Fv induced HEL-dependent cell growth of factor-dependent haematopoietic and hybridoma cell lines (*16*, *17*). This is based on the fact that the V_H/V_L interaction of HyHEL-10 is

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very weak ($K_a < 10^5$/M) in the absence of HEL, but can be significantly strengthened (to $K_a > 10^8$/M) in the presence of HEL, which can mimic activation of heterodimeric receptor chains upon binding with their cognate ligands (*18*). In addition, these chimeric receptors could be further applied to specifically amplify gene-transduced cells in an antigen-dependent manner (antigen-mediated genetically modified cell amplification, AMEGA) (*19*).

While these chimeric receptors are functional, it remains to be investigated exactly how they mimic signal transduction through the corresponding wild-type receptors. For example, growth properties, activation patterns and intensities of signal transducers remain to be solved. In this study, we compared the growth properties and phosphorylation events of intracellular signal transducers between EpoR and/or gp130-based chimeric receptor chains and wild-type EpoR and/or EpoR-gp130 chimera, respectively.

MATERIALS AND METHODS

Plasmid Construction—The construction of expression plasmids for V_H-EpoR (HE), V_L-EpoR (LE), V_H-gp130 (Hg) and V_L-gp130 (Lg) was described in previous studies (pME-HE, pMEZ-LE, pME-Hg and pMEZ-Lg, respectively) (*16*, *17*). To produce an expression plasmid for EpoR-gp130 chimera (Eg), pME-EpoR was digested with *Eco*RV and *Xba*I to delete transmembrane and intracellular domains, followed by ligation of murine gp130 transmembrane and intracellular domains derived from *Eco*RV- and *Xba*I-digested fragment of pME-Hg.

Cell Culture—A murine IL-3-dependent myeloid cell line, 32D (*20*, *21*), was cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (Iwaki, Tokyo, Japan) and 1 ng/ml of murine IL-3 (Genzyme/Techne, Cambridge, MA).

Transfection and Selection—To establish 32D/Hg + Lg cells, 32D cells (3×10^6) were washed and resuspended with 500 μl Hanks' buffered saline (Nissui Pharmaceutical), and mixed with 10 μg each of pME-Hg and pMEZ-Lg. The mixture was transferred into a cuvette, incubated for 10 min at room temperature, and electroporated once with Electroporator II (Invitrogen, Groningen, The Netherlands) set at 250 μF, 600 V. After 10-min incubation at room temperature, cells were transferred to 10-ml medium in 100-mm diameter culture dish and incubated at 37°C, 5% CO_2 for 2 days, followed by the selection with 480 μg/ml G418 (Sigma, St Louis, MO) and 500 μg/ml zeocin (Invitrogen). The drug-resistant cells were further cloned by limiting dilution. Likewise, 32D/HE + Lg, 32D/Hg + LE, 32D/EpoR, 32D/HE + LE and 32D/Eg cells were established by transfection of corresponding plasmids.

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 16,000*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 5% skimmed milk, the blot was probed with 1 : 1000 diluted primary rabbit antibody followed by 1 : 1000 diluted HRP-conjugated anti-rabbit IgG (Biosource, Camarillo, CA), and detection was performed using ECL system (Amersham-Pharmacia). Primary rabbit anti-mouse antibody used are: anti-EpoR C-terminus (Santa Cruz Biotechnology, Santa Cruz, CA), anti-gp130 C-terminus (Santa Cruz Biotechnology), anti-β tubulin (Santa Cruz Biotechnology), anti-phospho Stat3 (Santa Cruz Biotechnology), anti-phospho Stat5 (Cell Signaling Technologies, Danvers, MA), anti-phospho ERK (Promega, Madison, WI), anti-phospho Akt (Cell Signaling Technologies), anti-Stat3 (Santa Cruz Biotechnology), anti-Stat5 (Santa Cruz Biotechnology), anti-ERK (Santa Cruz Biotechnology) and anti-Akt (Cell Signaling Technologies).

Cell Growth Assay—Cells were washed twice with PBS and seeded in 24-well plates containing various concentrations of HEL (Seikagaku, Tokyo, Japan) or Epo (Kirin, Tokyo, Japan). The initial cell concentration was adjusted to 10^4 cells/ml. Viable cell concentrations were determined using a haemocytometer and the trypan blue exclusion assay.

Starvation and Stimulation of Cells—Cells were washed twice with PBS and starved in the depletion medium (RPMI1640, 10% FBS) for 12 h, 10^6 cells were stimulated with 500 μl medium containing various concentrations of HEL at 37°C, added with 1 ml ice-cold 3 mM Na_3VO_4 in PBS, pelleted and lysed with 100 μl of lysis buffer to prepare the lysate for western blot analysis.

RESULTS

Establishment of Transfectant Cell Lines—The combinations of chimeric receptor chains investigated in this study are shown in Fig. 1. Using a murine IL-3-dependent myeloid cell line 32D, we compared the signalling properties of Fv-EpoR chimera (HE + LE) and wild-type EpoR and those of Fv-gp130 chimera (Hg + Lg) and EpoR-gp130 (Eg) chimera, where the transmembrane and intracellular domains of EpoR was replaced with those of gp130, thereby mimicking wild-type gp130 signalling in response to Epo. As heterodimeric combinations of Fv-EpoR and Fv-gp130 chimeras (HE + Lg and Hg + LE) were functional in a pro-B cell line Ba/F3 (*16*, *19*), we also investigated the extent to which artificially induced heterodimers could mimic EpoR- and gp130-derived signals in another IL-3-dependent cell line 32D as a host.

Since the wild-type EpoR and HE + LE chimera are known to be functional in 32D cells (*17*), we first investigated whether Hg + Lg, Eg, HE + Lg or Hg + LE chimera could transduce a growth signal in 32D cells. The cells were electroporated with corresponding expression plasmids, followed by a drug-resistance selection to establish 32D/Hg + Lg, 32D/Eg, 32D/HE + Lg or 32D/Hg + LE cells, respectively. The cells were then washed with PBS to eliminate IL-3 and drugs from the culture media, and cultured in Epo (32D/Eg) or HEL (32D/Hg + Lg, 32D/HE + Lg and 32D/Hg + LE).

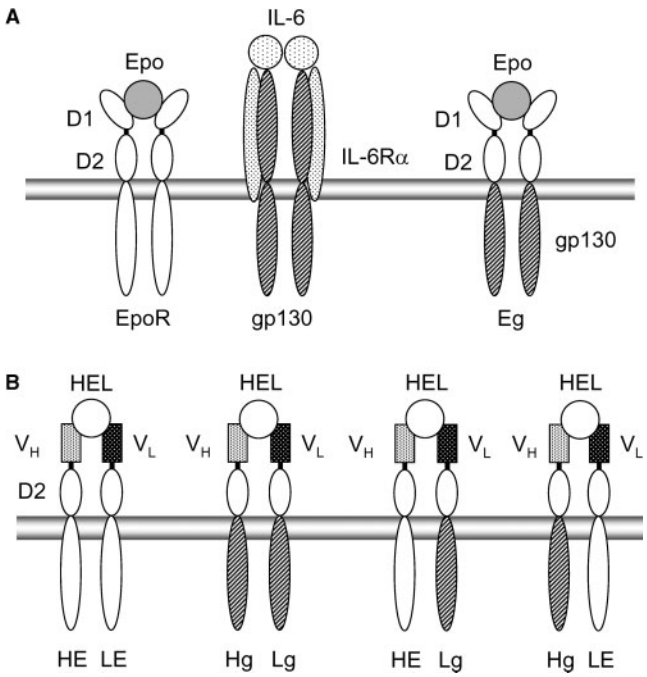

Fig. 1. **Schematic representations of chimeric receptors.** (A) Wild-type EpoR, gp130 and Epo-responsive Eg chimera. The transmembrane and intracellular domains of wild-type EpoR were replaced with those of gp130 to create Eg chimera. (B) HEL-responsive chimeric receptors. HE and LE chimeric receptors were constructed by replacing the extracellular D1 domain of EpoR with V_H and V_L regions of anti-HEL antibody HyHEL-10. Hg and Lg were generated by replacing the

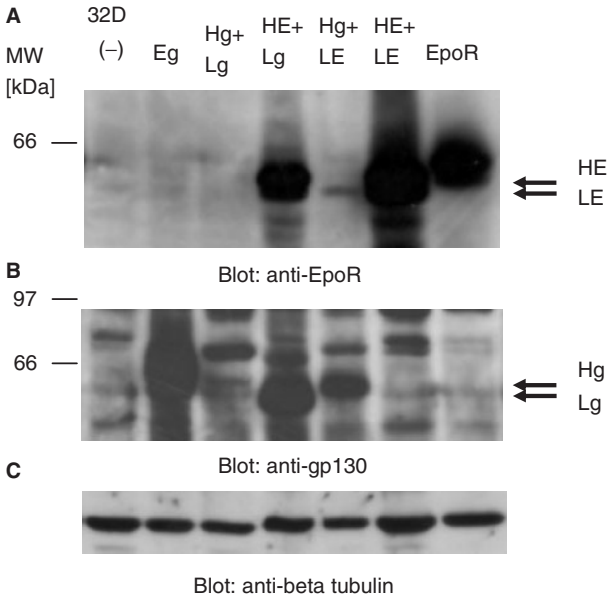

Fig. 2. **Expression of the chimeric receptors.** The cell lysates were subjected to Western blot analysis using anti-gp130 or anti-EpoR C-terminus antibody. The reprobed blots with anti-β tubulin antibody are shown as a loading control. Parental 32D cell lysate was used as a negative control.

Consequently, all transfectants grew in response to the corresponding ligand. To confirm the expression of chimeric receptors, the lysates from the transfectants were subjected to western blot analysis (Fig. 2). All the transfectants expressed corresponding chimera, although the expression levels of both Hg and Lg in 32D/Hg + Lg, and LE in 32D/Hg + LE were lower than those of other chimeras. These results indicate that we successfully established transfectants of a series of chimeric receptors for further analyses.

It is noteworthy that there appeared an intense band shifted to a higher molecular weight around 70 kDa in the lanes for 32D/Hg + Lg, 32D/HE + Lg and 32D/Hg + LE. The bands were different from the two (probably non-specific) bands around 70 kDa seen in 32D, and in particular 32D/HE + LE cells, in which a larger amount of total protein was loaded as suggested by the anti-tubulin blot. The results indicate that these bands are derived from Hg and/or Lg, and might contribute to promote cell growth especially in 32D/Hg + Lg cells, although the reason for the mobility shift remains unclear. Another possibility is that the 70 kDa band is non-productive and the threshold of Hg/Lg expression for enabling cell growth would be low enough to accept considerably low expression levels of the chimeras in this experimental system.

Growth Signal from Chimeric Receptors—To investigate whether the growth of the transfectant cell lines can be stimulated by the corresponding ligand supplemented to the IL-3-deficient medium, cells were washed and cultured in various concentrations of Epo or HEL for 7 days, and the time-course of viable cell concentration was plotted. As expected, 32D/Eg and 32D/Hg + Lg cells proliferated in an Epo- and HEL-dependent manner, respectively (Fig. 3), while the growth rate of 32D/Eg was faster than that of 32D/Hg + Lg. Interestingly, 32D/HE + Lg and 32D/Hg + LE cells also showed clear and efficient HEL-dependent cell growth (Fig. 4). In this case, the growth speed of 32D/HE + Lg was faster than that of 32D/Hg + LE, which might be attributed to reduced expression of LE chain (see Discussion section). These results indicate that chimeric receptor chains, inducing a cytoplasmic EpoR-gp130 heterodimer as well as gp130 homodimer, can transduce a growth signal in 32D cells.

Comparison of Signalling Properties from Chimeric Receptors—The growth assay revealed that antibody/receptor chimeras mimicked the wild-type receptor chimeras with regard to the long-term growth properties. To investigate whether this mimicry was also observed for shorter-term signal transduction events, the phosphorylation of key signalling molecules was compared. Here we focussed on Stat3, Stat5, ERK and Akt, as the Jak/Stat, Ras/MAPK and PI3K/Akt pathways are considered to be major pathways for cytokine signalling. To distinguish gp130-derived signalling from EpoR-derived signalling, we selected two Stats, since Stat3 and Stat5 are mainly activated by gp130 and EpoR, respectively (*22–24*). The cells were starved in a ligand-deficient medium, and stimulated with Epo, HEL or IL-3, followed by western blot analysis using antibodies against phosphorylated signalling molecules. In parental 32D cells, HEL did not induce phosphorylation of any molecules, while Epo induced very weak Stat5

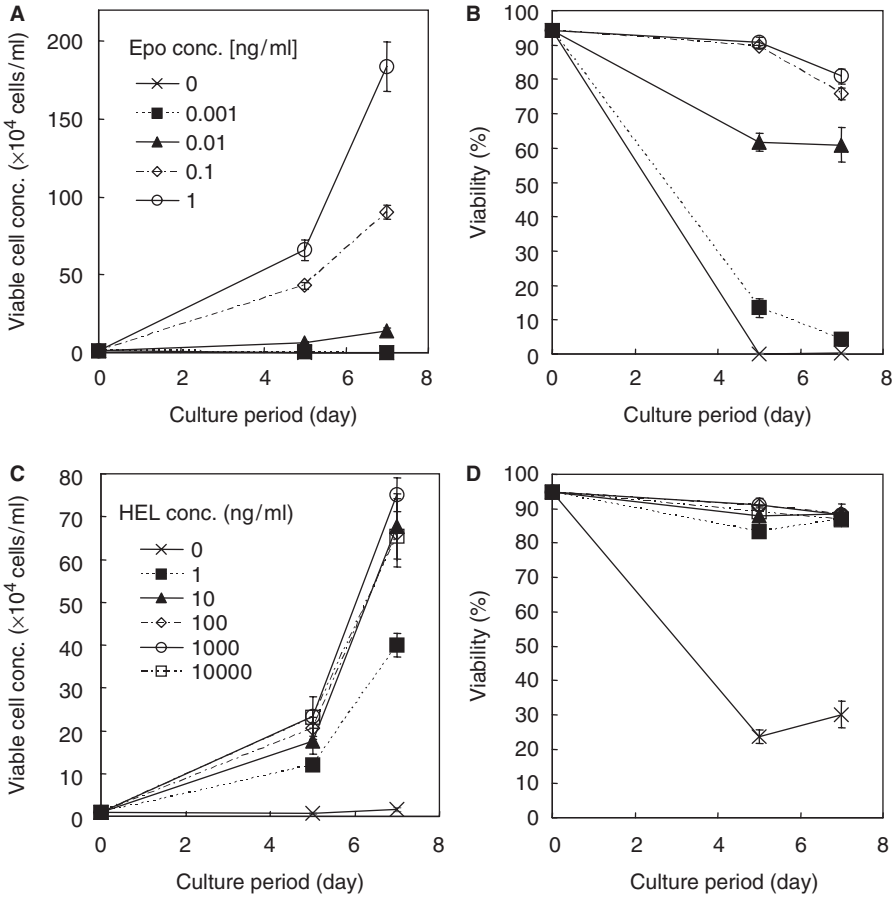

Fig. 3. **Cell growth properties of 32D/Eg and 32D/Hg + Lg cells.** Cells (10^4 cells/ml) were inoculated into 24-well plates at day 0, and cultured in the presence of Epo or HEL at several concentrations. Viable cell concentration and viability are plotted as the mean plus standard deviation of three individual tests. (A) Epo-dependent growth of 32D/Eg cells. (B) Viability of 32D/Eg cells, where the keys are as in (A). (C) HEL-dependent growth of 32D/Hg + Lg cells. (D) Viability of 32D/Hg + Lg cells, where the keys are as in (C).

phosphorylation (Fig. 5). In comparison, IL-3 induced weak phosphorylation of Stat3 and strong phosphorylation of Stat5, ERK and Akt.

We first compared the signalling properties of gp130-based chimeras (Fig. 5). 32D/Eg cells exhibited phosphorylation of all the four signalling molecules in a clear Epo-dependent manner. The level of Epo-induced Stat3 phosphorylation was much higher than IL-3-induced Stat3 phosphorylation, whereas Epo-induced phosphorylation of Stat5, ERK and Akt was less than that induced by IL-3. On the other hand, 32D/Hg + Lg cells exhibited ligand-independent phosphorylation for all the four signalling molecules even in the absence of HEL, although HEL addition seemed to stimulate phosphorylation of Stat3 and ERK to a little extent. The maximal phosphorylation levels of Stat3, ERK and Akt in 32D/Hg + Lg cells were comparable to those in 32D/Eg cells, while the phosphorylation level of Stat5 in 32D/Hg + Lg cells was significantly less than that in 32D/Eg cells.

To compare the signalling properties of antibody/EpoR chimera and wild-type EpoR, we performed similar experiment with 32D/EpoR and 32D/HE + LE cells (Fig. 6). For these cells, phosphorylation of Stat3 and MAPK was too weak to detect. While 32D/EpoR cells showed ligand-dependent phosphorylation of Stat5 and Akt, 32D/HE + LE cells exhibited ligand-independent phosphorylation (Fig. 6). The maximal phosphorylation levels of Stat5 and Akt in 32D/HE + LE cells were less than those in 32D/EpoR cells.

To investigate whether the heterodimeric combinations (HE + Lg and Hg + LE) share both EpoR and gp130 signalling properties, 32D/HE + Lg and 32D/Hg + LE cells were analysed (Fig. 7). As expected, both Stat3 and Stat5 were strongly phosphorylated in both 32D/HE + Lg and 32D/Hg + LE cells in a ligand-independent manner. Like wild-type EpoR and the EpoR-based chimera (HE + LE), ERK phosphorylation was weak in these two cells. Akt phosphorylation was also observed like the EpoR- and gp130-based chimeras. Therefore, HE + Lg and Hg + LE chimeras can transduce both EpoR- and gp130-derived signals.

These results suggest that the chimeric receptors can activate the same set of signalling molecules as wild-type receptors, although the chimeric receptors showed ligand-independency and a little lower maximal

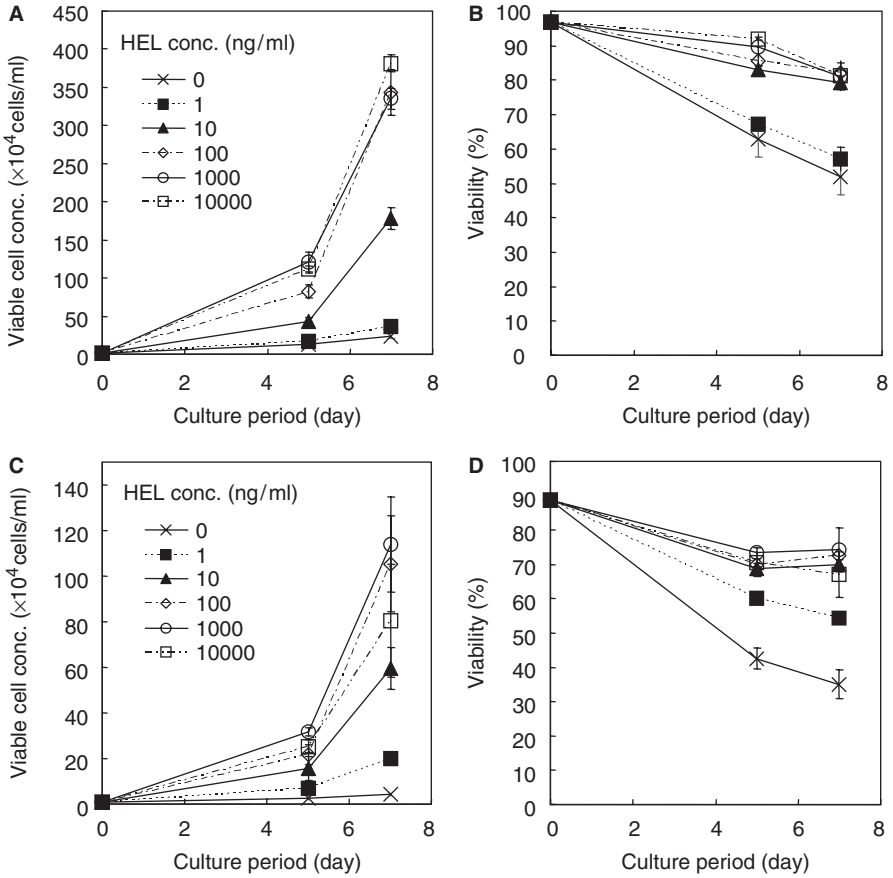

Fig. 4. **Cell growth properties of 32D/HE + Lg and 32D/Hg + LE cells.** Cells (10^4 cells/ml) were inoculated into 24-well plates at day 0, and cultured in the presence of HEL at several concentrations. Viable cell concentration and viability are plotted as the mean plus standard deviation of three individual tests. (A) HEL-dependent growth of 32D/HE + Lg cells. (B) Viability of 32D/HE + Lg cells, where the keys are as in (A). (C) HEL-dependent growth of 32D/Hg + LE cells. (D) Viability of 32D/Hg + LE cells, where the keys are as in (C).

phosphorylation than the corresponding wild-type receptors.

DISCUSSION

In this study, we demonstrated mimicry of erythropoietin and interleukin-6 signalling by antibody/cytokine receptor chimera in murine myeloid 32D cells. As in Ba/F3 cells, EpoR-gp130 cytoplasmic domain heterodimer combinations were also found to be functional in this cell line. A previous study reported that ectopic expression of IL-6R in 32D cells could transduce only a short-term growth signal in response to IL-6 (*25*). In addition, a recent study showed that an EpoR mutant with Stat3 binding motifs, which were derived from gp130, led to Stat3 activation, followed by growth arrest of 32D cells (*24*). In this study, we found that gp130-derived signals with significant Stat3 activation could support long-term growth of 32D cells. This apparent difference might be due to the difference in receptor constructs, conformations and/or expression levels, thereby inducing distinct patterns of activation for many signalling molecules involved in gp130 signalling pathway.

In the western blot to detect gp130 cytoplasmic domain, 32D/Hg + Lg cells showed two faint bands around 58 kDa and another band shifted to a higher molecular weight around 70 kDa. The band around 70 kDa was also observed in 32D/HE + Lg and 32D/Hg + LE cells. We speculate that the band around 70 kDa might correspond to the activated state of the chimeras. The band shifts might be due to tyrosine phosphorylation and/or dimerization of the receptor chains, which was retained even in the denaturing and reducing condition. Such a dimer could be formed by a non-covalent association between Hg and Lg chains, or more likely, by cysteine residues in the transmembrane domain of gp130 that were insufficiently reduced and/or cross-linked again with the nearest cysteine after once reduced. The other possibility is a tight binding of another molecule that is specific for and barely dissociate from the Hg + Lg chimera. Further molecular characterization of this phenomenon might lead to a better understanding of the activation mechanism for the chimeras.

We initially expected that the conformational difference between HE + Lg and Hg + LE chimeras might

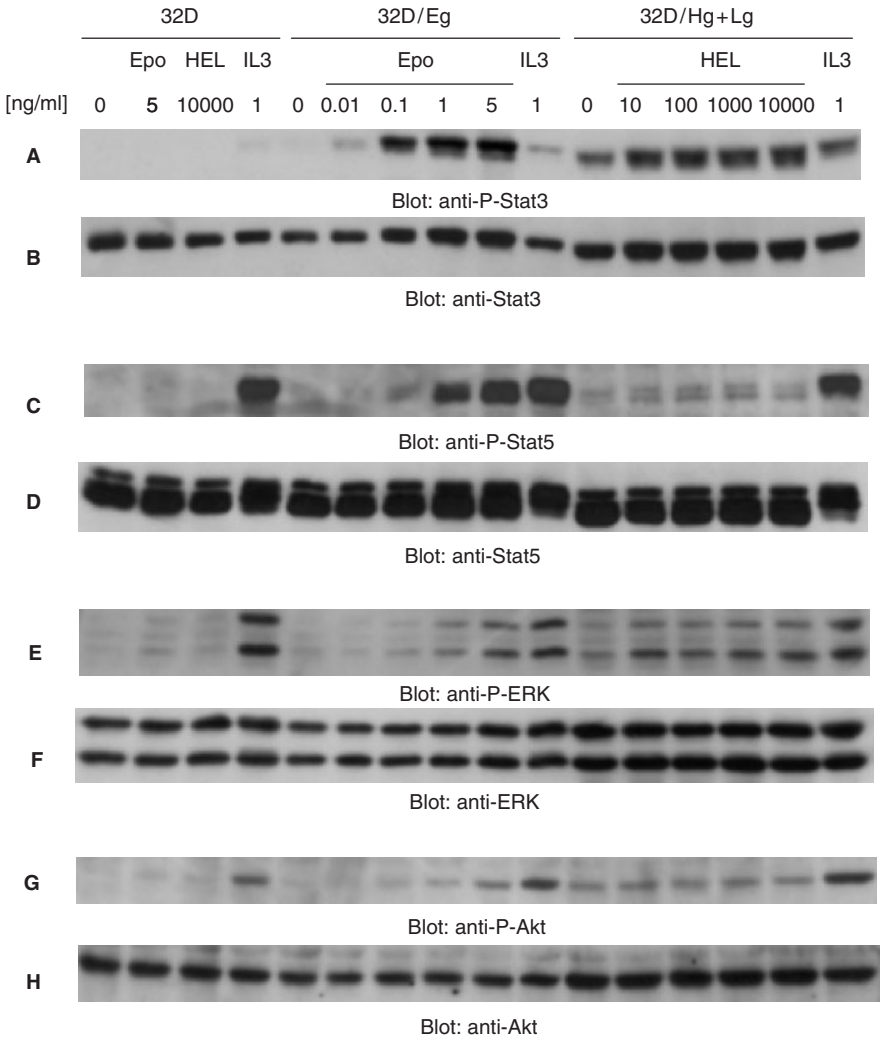

Fig. 5. **Signal transduction properties in 32D/Eg and 32D/Hg+Lg cells.** Cells (7.5×10^4 cells) were washed with PBS twice and stimulated with or without ligand for 15 min at 37°C, supplemented with ice-cold 1 mM Na_3VO_4 in PBS, pelleted and then lysed. The lysate was subjected to western blot analysis with anti-phospho Stat3 antibody (A), anti-phospho Stat5 antibody (C), anti-phospho ERK antibody (E) or anti-phospho Akt antibody (G), and followed by reprobing with anti-STAT3 antibody (B), anti-Stat5 antibody (D), anti-ERK antibody (F) or anti-Akt antibody (H), respectively.

affect the activation pattern of the downstream signalling molecules. In fact, Stat5 phosphorylation, which is mainly derived from EpoR signalling, was significantly reduced in the Hg+LE chimera compared to HE+Lg chimera, whereas phosphorylation of the other signalling molecules remains almost comparable. However, since the signalling should be also affected by receptor expression levels as well as the conformational effect, the difference between HE+Lg and Hg+LE observed here might be due to the differential expression levels of the chimeras, rather than conformational effect. Establishing transfectants with similar expression levels of chimeras might reveal a conformational effect for heterodimeric receptor combinations.

While the wild-type EpoR and Eg chimera showed a clear Epo-dependent growth signal, our antibody/receptor chimeras showed weaker HEL-dependency. Since the phosphorylation levels of the signalling molecules in the presence of the cognate ligand were comparable between Epo-responsive and HEL-responsive receptors, the difference of ligand-dependency is mainly due to the difference in the unliganded states. According to an X-ray crystallographic analysis, unliganded EpoR forms a dimer through the interaction between D1 domains of each receptor chain, while keeping the receptor dimer incompetent for signalling (*2*). This may indicate that the D1 domain is critical, not only for ligand binding, but also for maintenance of a switched-off conformational state of unliganded EpoR. In our chimeric receptors, we replaced EpoR D1 domain with an antibody variable region, which has a different tertiary structure to the EpoR D1 domain. Furthermore, although the binding affinity between V_H and V_L of HyHEL-10 was $<10^5$/M by surface plasmon resonance analysis (*18*), the actual binding affinity, based on the bound-free equilibrium on the plasma membrane where only lateral diffusion is possible, could be much stronger. Therefore, it would be surprising that our chimeric receptors retained

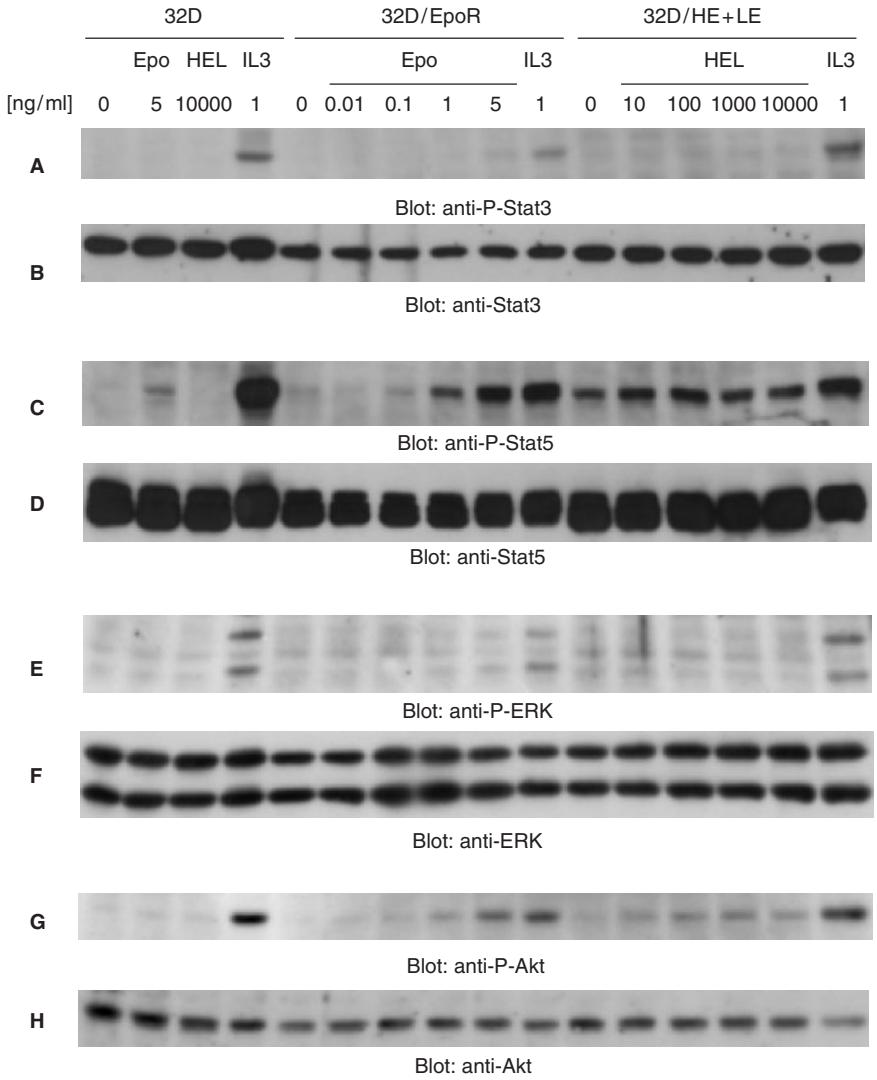

Fig. 6. **Signal transduction properties in 32D/EpoR and 32D/HE + LE cells.** Cells (7.5×10^4 cells) were washed with PBS twice and stimulated with or without ligand for 15 min at 37°C, supplemented with ice-cold 1 mM Na_3VO_4 in PBS, pelleted and then lysed. The lysate was subjected to western blot analysis with anti-phospho Stat3 antibody (A), anti-phospho Stat5 antibody (C), anti-phospho ERK antibody (E) or anti-phospho Akt antibody (G), and followed by reprobing with anti-STAT3 antibody (B), anti-Stat5 antibody (D), anti-ERK antibody (F) or anti-Akt antibody (H), respectively.

substantially similar signalling properties to those of the wild-type receptor. The ligand-independent signalling in the antibody/receptor chimeras might suggest some conformational differences between the wild-type and antibody/receptor chimeras, especially in the unliganded states. In other words, mimicry of cytokine receptor by antibody/receptor chimera would be attained by complete mimicry of the switched-off state. In this sense, it is of quite interest how the wild-type receptor prevents a constitutively active conformational state in the absence of their cognate ligand.

It was also unexpected that the phosphorylation events by antibody/receptor chimeras were almost independent of the stimulation with HEL, which contradicts the results from the growth assay showing HEL-dependent cell growth. This indicates that short-term phosphorylation events could not exactly reflect the long-term growth properties for the chimeras, and/or other signalling molecules might be involved in the HEL-dependent cell growth. The results presented in this study indicate that additional molecular design for antibody/receptor chimeras might be required to attain more precise mimicry of wild-type receptors. A recent report revealed that two simultaneous mutations (L241G and L242P) to introduce a kink into the leucine zipper motif in the EpoR transmembrane domain resulted in a marked decrease of interchain interaction of EpoR, thereby leading to a decreased intensity of the growth signal (*26*). Furthermore, the mutagenesis in the juxtamembrane region of EpoR and gp130 revealed that orientation of the cytoplasmic domain is critical for signal transduction (*27–29*). In addition, a subtle change in the antigen-binding region could turn the same antigen from agonists to even antagonists, as shown in our previous study (*30*). Since these results indicate the susceptible feature of cytokine receptor to subtle mutations, we

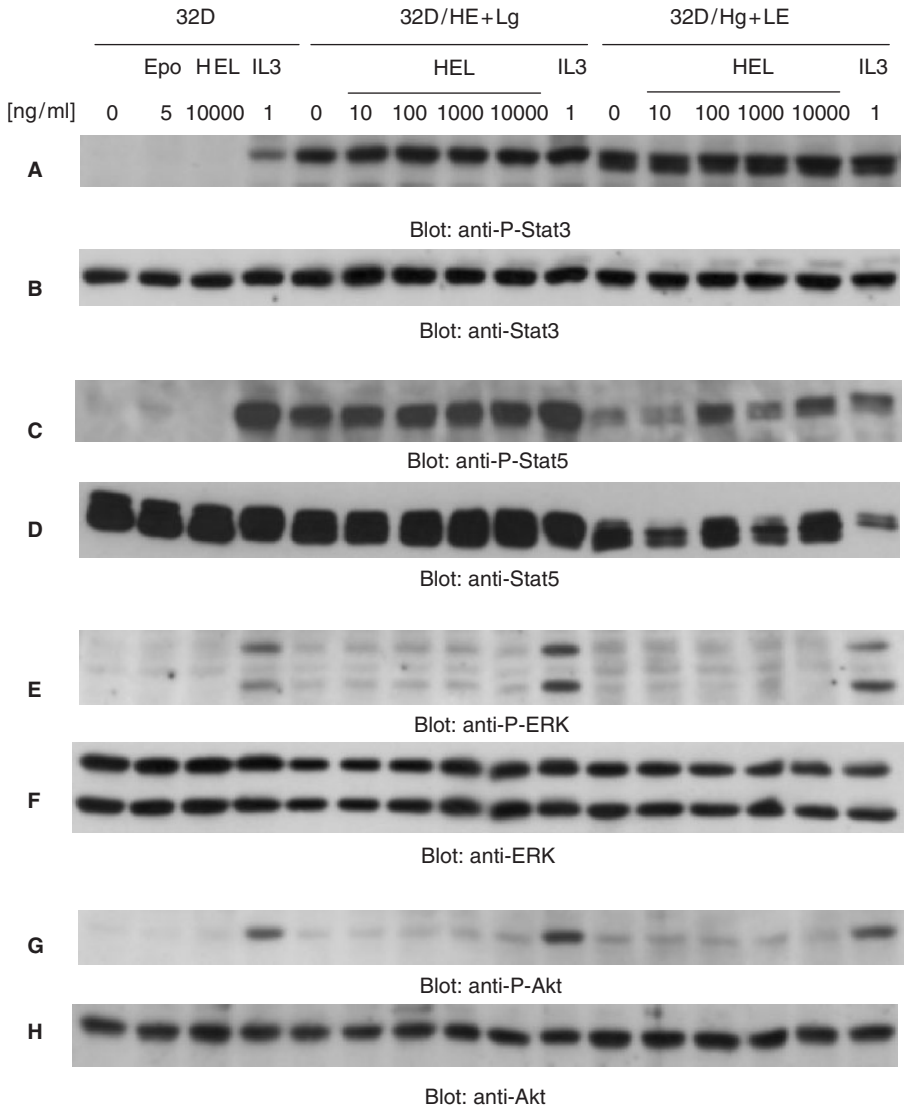

Fig. 7. **Signal transduction properties in 32D/HE + Lg and 32D/Hg + LE cells.** Cells (7.5×10^4 cells) were washed with PBS twice and stimulated with or without ligand for 15 min at 37°C, supplemented with ice-cold 1 mM Na_3VO_4 in PBS, pelleted and then lysed. The lysate was subjected to western blot analysis with anti-phospho Stat3 antibody (A), anti-phospho Stat5 antibody (C), anti-phospho ERK antibody (E) or anti-phospho Akt antibody (G), and followed by reprobing with anti-STAT3 antibody (B), anti-Stat5 antibody (D), anti-ERK antibody (F) or anti-Akt antibody (H), respectively.

believe that the combinations of these modifications could attain additional fine-tuning of the antibody/receptor chimeras. Further molecular design towards the maximal mimicry of wild-type receptors would certainly contribute to the understanding of cytokine receptor biology, as well as the receptor engineering for arbitrary specificities.

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