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The Influence of Domain Structures on the Signal Transduction of Chimeric Receptors Derived from the Erythropoietin Receptor

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Although cytokine receptors regulate many cellular functions, contribution of receptor's domains and their conformation to signal transduction remains unclear. In this study, we designed a series of chimeric erythropoietin receptor (EpoR) variants encoding a haemagglutinin epitope-tagged anti-fluorescein single-chain Fv and different combinations of extracellular D1/D2 domain(s) of EpoR as the extracellular domain to allow the receptor to be activated by multiple ligands. Furthermore, one to four Ala residues were inserted at the intracellular juxtamembrane region of each chimeric receptor to modulate the conformation of the intracellular domain. When the chimeric receptors were expressed in Ba/F3 cells, cell-surface expression levels of chimeric receptors without D2 domain were markedly lowered, suggesting a role of D2 domain for stabilizing the receptor. Furthermore, the ligand-dependent cell proliferation was strongly affected by extracellular domain structures and the number of inserted Ala residues. Moreover, the conformational change of chimeric receptors was induced by various ligands to detect the phosphorylation of JAK2, STAT5 and ERK2, whose activations are characteristics of EpoR signalling. Consequently, the phosphorylation pattern of these signal transducers was significantly influenced by ligands and receptor variants. These results indicate that signal transduction of EpoR is strongly affected by conformation of both extracellular and intracellular domains.

Key words: chimeric receptor, conformational change, erythropoietin receptor, ligand, signal transduction.

Abbreviations: BSA-FL, fluorescein-conjugated BSA; EGFP, enhanced green fluorescent protein; EMP, Epo-mimetic peptides; EpoR, erythropoietin receptor; ERK2, the extracellular signal-regulated kinase 2; HA, haemagglutinin; IL-3, interleukin-3; JAK2, Janus kinase 2; STAT5, signal transducer and activator of transcription 5; TM, transmembrane.

Cytokines are small, secretory proteins and their main function is to control cell fate. Cytokines bind to their cognate receptors expressed on the surface of target cells, which trigger conformational change or oligomerization of the receptors to activate the downstream signalling cascade (*1*). The erythropoietin receptor (EpoR) belongs to the cytokine receptor superfamily and is the primary regulator of mammalian erythropoiesis (*2*, *3*). The extracellular D1 domains of EpoR exist as a preformed dimer in an open scissor-like conformation in the absence of ligand (*4*). Epo binding to the D1 domains induces a conformational change, which activates the pre-bound cytoplasmic tyrosine kinase, Janus kinase 2 (JAK2) (*5*, *6*). JAK2 is essential for signalling by receptors for growth hormone, prolactin, erythropoietin, thrombopoietin, interleukin (IL)-3 and IL-5. The main downstream signalling molecules are the signal transducer and activator of transcription 5 (STAT5), Ras/MAPK and PI3K/AKT pathways (*7*). In EpoR, activated JAK2 phosphorylates several tyrosine residues in the cytoplasmic tail that acts as docking sites for signalling molecules containing Src-homology 2 (SH2) domains.

Numerous studies have examined the roles of extracellular, transmembrane (TM) and intracellular domains of the wild-type EpoR. Instead of Epo, oligopeptide dimers named Epo-mimetic peptides (EMP) were reported to bind to the extracellular domain of EpoR (*8*, *9*). The structures of two EMP derivatives, EMP1 and EMP33, show high similarity in terms of both main- and side-chain conformations. However, X-ray crystallographic analyses of EMP1-EpoR and EMP33-EpoR complexes revealed that small differences in the orientation of the extracellular domain of EpoR determine whether these peptide ligands act as either an agonist (EMP1) or an antagonist (EMP33) (*8*, *9*). This result suggests that signal transduction is strongly affected by the conformational change of the extracellular domains. In addition, several studies have demonstrated that the TM domain of cytokine receptors is a key determinant for oligomerization (*10*–*12*). The oligomerization activity of the EpoR TM has been found to be very high, as determined using a bacterial reporter system (*13*). In addition, there have been studies on the influence of orientational

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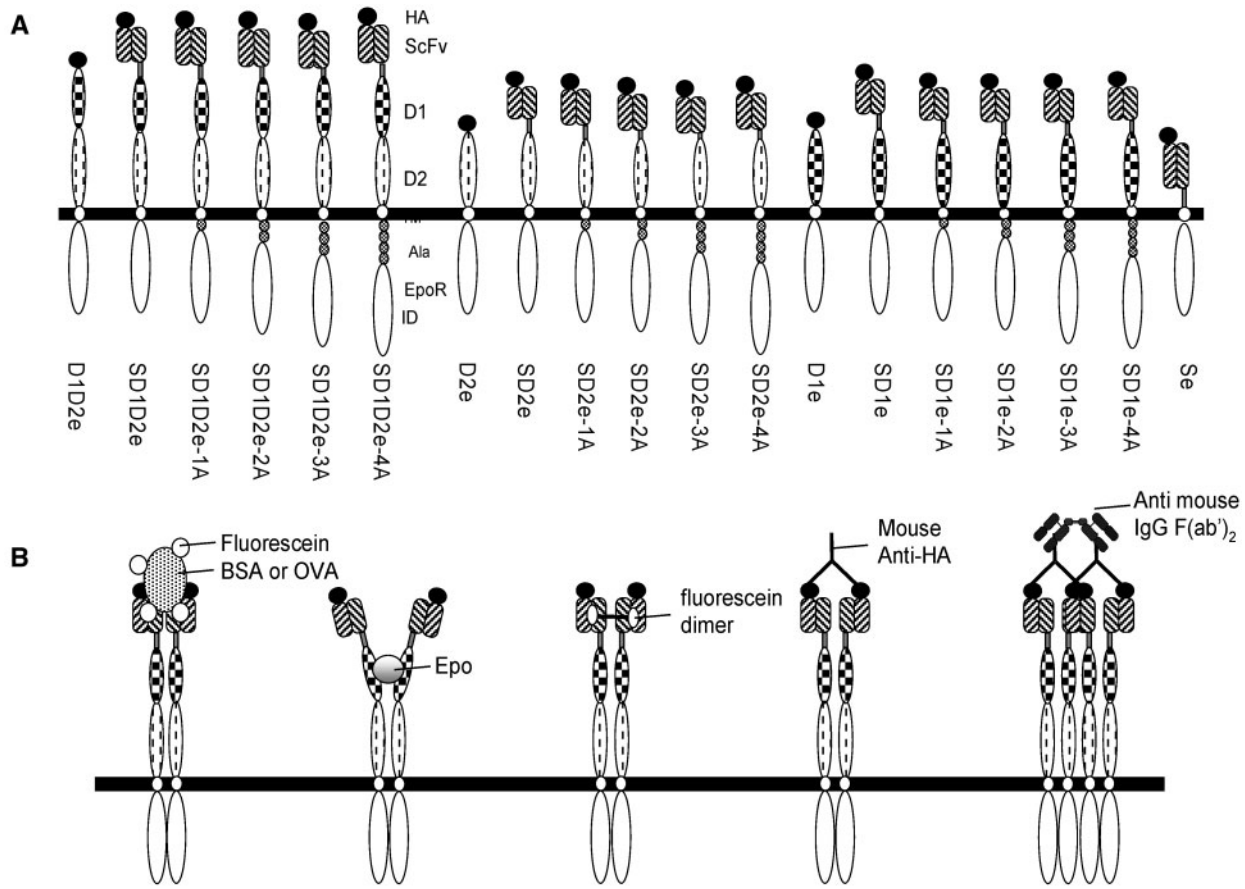

Fig. 1. **Schematic diagrams of the chimeric receptor constructs.** (A) A series of chimeric receptor constructs with different combinations of the domains containing the anti-FL ScFv clone 31IJ3, extracellular D1/D2, TM and intracellular domains of EpoR. An HA-tag was fused to the extracellular N-terminus of each chimeric receptor. (B) Schematic diagram of the ligand binding to chimeric receptors. BSA-FL and FL dimer-13 could bind to the ScFv region of the chimeric receptors. Epo could bind to the extracellular D1 domain of the chimeric receptors. A mouse anti-HA antibody could bind to the HA-tag fused to the N-terminus of the chimeric receptors. Furthermore, addition of anti-mouse IgG antibody together with the mouse anti-HA antibody could induce oligomerization of the chimeric receptors.

changes of the EpoR intracellular domain on signal transduction (*14*). For example, Constantinescu *et al*. (*14*) evaluated the effect of alanine-insertion mutagenesis in the intracellular juxtamembrane domain of EpoR, in which each extra residue rotates the register of a predicted α helix by 109°. They showed that EpoR mutants with one or four additional Ala residues had markedly impaired signalling for cell proliferation, while an EpoR mutant with three additional Ala residues signalling equivalent to the wild-type EpoR. More recently, Greiser *et al*. (*15*) also reported a similar observation for an IL-5R/gp130 chimera, showing that the orientational change of the gp130 intracellular domain by inserting one to four Ala residues at the intracellular juxtamembrane domain strongly affects the activation of STAT1 and STAT3.

Although these studies clearly demonstrated the influence of the orientational change of the intracellular domain on signal transduction, they did not examine the influence of the extracellular domains on signal transduction. Therefore, in this study, we designed a series of antibody/EpoR chimeras containing a haemagglutinin (HA)-tagged anti-fluorescein ScFv tethered to different combinations of EpoR D1/D2 domains in the extracellular domain (Fig. 1A). Such chimeras could recognize multiple ligands in the same receptor chain (Fig. 1B). Fluorescein derivatives such as fluorescein-conjugated BSA (BSA-FL) and a fluorescein dimer connected with palindromic 13-mer DNA (FL dimer-13) could bind to the ScFv region of the chimeric receptors. Epo could bind to the extracellular D1 domain in the chimeric receptors. A mouse anti-HA antibody could bind to the HA-tag fused to the N terminus of the chimeric receptors, and could be used as a dimerizer (*16*). Furthermore, the addition of anti-mouse IgG antibody together with the mouse anti-HA antibody could further induce oligomerization of the chimeric receptors. Since these ligands could induce distinct conformations of the extracellular domain of each chimeric receptor, much information would be obtained with regard to the role of the extracellular domain of EpoR. Furthermore, we also inserted one to four Ala residues at the intracellular juxtamembrane region of each chimeric receptor to modulate the orientation of the intracellular domain. Overall, such receptor engineering would offer more systematic analysis to elucidate the importance of conformation in EpoR.

To test the signal-transduction ability of each chimeric receptor, the IL-3-dependent pro-B cell line Ba/F3 was transduced with a vector encoding each chimeric receptor. The transduced cells were stimulated with a series of ligands to examine the ligand-dependent cell growth and the phosphorylation levels of signal transducer proteins such as JAK2, STAT5 and the extracellular signal-regulated kinase 2 (ERK2). Using these techniques, we evaluated the influence of the individual combinations of extracellular and intracellular domains on the signal transduction in EpoR.

EXPERIMENTAL PROCEDURES

Vector Construction—A series of chimeric receptor constructs with different combinations of the domains containing an anti-FL ScFv clone of 31IJ3, extracellular D1/D2 domains, TM and intracellular domain of human EpoR were constructed. To simplify the names of the constructs, abbreviation was used as follows: S, single chain Fv; D1, EpoR extracellular D1 domain; D2, EpoR extracellular D2 domain; e, EpoR intracellular domain. The number of Ala residues inserted between TM and intracellular domains was also indicated. For example, chimeric receptor SD1D2e-2A encodes ScFv-D1-D2 as the extracellular domain, EpoR intracellular domain and two Ala residues were inserted between TM and intracellular domain.

The constructions of vectors encoding D1D2e, SD1D2e, D1e, SD1e, D2e, SD2e and Se were as described (*17*). To insert Ala residues between the EpoR TM and intracellular domain, the fragment containing one to four Ala residues and the intracellular domain of EpoR was amplified with PCR using five primers (KiTM 1A for: 5′-GGGCGTACGGCTTCCCACCGCCGGGCTCTG-3′, KiTM 2A For: 5′-GGGCGTACGGCTGCCTCCCACCGC CGGGCTCTG-3′, KiTM 3A For: 5′-GGGCGTACGGCTG CCGCATCCCACCGCCGGGCTCTG-3′, KiTM 4A For: 5′-GGGCGTACGGCTGCCGCAGCGTCCCACCGCCGGG CTCTG-3′ and KiTM A Rev: 5′-CGTTAGGGGGGGGGGA GGGAGAGGGGCGGATCCATCG-3′) and SD1D2e as a template. The amplified fragment was digested with *Bsi*WI (underlined) and *Bam*HI (double underlined) and subcloned into SD1D2e, SD1e and SD2e that were digested with *Bsi*WI and *Bam*HI to make SD1D2e-1A to SD1D2e-4A, SD1e-1A to SD1e-4A and SD2e-1A to SD2e-4A, respectively. In this study, all of the fragments amplified with PCR were confirmed for their sequence using a SQ-5500 Sequencer (Hitachi, Tokyo) and a Thermosequenase sequencing kit (Amersham, Buckinghamshire, UK).

The chimeric receptor cDNA was cloned in the pMK-IRES-EGFP bicistronic retroviral vector upstream of internal ribosomal entry site (IRES). The IRES sequence is used to couple the expression levels of the two cistrons, and placed downstream of chimeric receptor and upstream of the enhanced green fluorescent protein (EGFP). Here, EGFP was used as a marker gene, since FACS analysis readily shows the transduction/selection efficiencies.

Cell Lines—Ba/F3, a lymphoid cell line dependent on IL-3 for survival and proliferation, was cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo) supplemented with 10% FBS (BioWest, France), 4 mM L-Glutamine (Sigma) and 1 ng/ml murine IL-3 (Genzyme, Cambridge, MA, USA) at 37°C in a 5% CO_2 incubator. A retroviral packaging cell line, Plat-E, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 4 mM L-Glutamine, 1 μg/ml puromycin (Sigma) and 10 μg/ml blasticidin (Kaken Pharmaceutical, Japan).

Retroviral Production and Transduction—Plat-E cells were inoculated into a 60 mm diameter dish at 5×10^5 cells/ml in 4 ml of DMEM containing 10% FBS and cultured for 20 h. Plat-E cells were transfected with a vector using Lipofectamine reagent (Invitrogen) and Plus reagent (Invitrogen). One microgram of each vector was sterilized by ethanol precipitation, and solubilized in 125 μl serum-free DMEM with 20 μl Plus reagent. After incubation at room temperature for 15–45 min, 125 μl serum-free DMEM with 5 μl Lipofectamine reagent was mixed with the DNA-Plus reagent solution and incubated at room temperature for 15–45 min. The cells were washed once with 2 ml serum- and antibiotics-free DMEM, and 1 ml serum-free DMEM was added to each 60 mm diameter dish. The Lipofectamine–DNA complex solution was overlaid onto the washed cells. After incubation of the cells for 3–5 h at 37°C in a 5% CO_2 incubator, the medium was replaced with a normal growth medium. The medium was further replaced after 24 h. After additional 24 h incubation, Ba/F3 cells (2×10^5 per well) were transduced with the retroviral virus-containing conditioned media in a 24-well plate by using RetroNectin (Takara-Bio).

Western Blotting—The EGFP-positive cells were used for western blotting. The cells (1×10^6) were washed with PBS, lysed with 100 μl of lysis buffer [20 mM HEPES (pH 7.5), 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] and incubated on ice for 10 min. After centrifugation at 15,000 r.p.m. in a KUBOTA RA-50J rotor (KUBOTA, Japan) at room temperature for 10 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, USA). After the membrane was blocked with Blocking One (Nakarai Tesque, Kyoto, Japan) for the detection of JAK2, STAT5 and ERK2 or with Blocking One-P (Nakarai Tesque) for the detection of phosphorylated JAK2, STAT5 and ERK2, the blot was probed with rabbit anti-mouse JAK2 (Upstate Biotechnology), rabbit anti-Phospho-Tyr1007/1008-JAK2 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-mouse STAT5 (Santa Cruz Biotechnology), rabbit anti-Phospho-Tyr694-Stat5 (Cell Signaling Technology), rabbit anti-mouse ERK (Santa Cruz Biotechnology) or an anti-ACTIVE MAPKpAb (Promega Corp, Madison, WI, USA) specific for tyrosine phosphorylated forms of ERK 1 and ERK 2, followed by HRP-conjugated anti-rabbit IgG (Biosource, Camarillo, CA, USA), and detection was performed using the ECL system (GE Healthcare). Band intensities were determined using ImageJ software from the National Institutes of Health (rsb.info.nih.gov/ij/docs/intro.html), which calculates

pixel value statistics of user-defined areas. Phosphorylation levels induced with each ligand were normalized by the respective constitutive expression levels and by levels obtained with IL-3 for each chimeric receptor.

Cell Proliferation Assay—The EGFP-positive cells with each chimeric receptor were washed three times with PBS and seeded in 96-well plates in 100 µl per well containing various concentrations of BSA (Sigma), BSA-FL (Sigma), fluorescein sodium salt (free FL; Sigma), Ovalbumin (OVA, Imject Ovalbumin; Pierce Biotechnology, Rockford, IL, USA) and fluorescein-conjugated OVA (OVA-FL, Molecular Probes, Eugene, OR, USA). Viable cell concentrations were determined using a Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). After several days of culture, 10 µl of CCK-8 per well was added and incubated for 4 h at 37°C in a CO_2 incubator. The cell concentrations in triplicate wells were estimated from the absorbance (450 nm) of reduced WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt; Dojindo] using a standard curve.

Surface Expression of HA-tagged Chimeric Receptors—Surface expression levels of HA-tagged chimeric receptors were measured with flow cytometry. Briefly, the EGFP-positive cells cultured in IL-3 were incubated for 30 min in cold PBS containing 2% BSA and 4% donkey serum (Buffer A). Cells were then incubated for 1 h with 67 nM mouse monoclonal anti-HA antibody diluted in Buffer A (Babco, Richmond, CA, USA), washed three times with PBS containing 0.2% BSA and 0.4% donkey serum (Buffer B), and incubated with R-phycoerythrin (PE)-conjugated donkey $F(ab')_2$ anti-mouse IgG secondary antibody (125 nM) in Buffer A at 4°C. Cells were washed three times with Buffer B and analysed with FACS Calibur flow cytometer (Becton-Dickinson, Lexington, KY, USA) with excitation at 488 nm and fluorescence detection at 585 ± 21 nm. Median fluorescence intensities of PE were used for quantitation since this measure of central tendency is least sensitive to outliers.

Stimulation Experiment—The EGFP-positive cells were washed three times and resuspended in RPMI medium, starved at 37°C for 12 h, and then stimulated with 67 pM IL-3, 76 nM BSA-FL, 164 pM Epo, 400 nM FL dimer-13, 67 nM mouse anti-HA antibody or 67 nM mouse anti-HA antibody plus 125 nM anti-mouse IgG. After stimulation at 37°C for 15 min, equal volume of ice-chilled PBS buffer with 2 mM Na_3VO_4 was added to cells to inhibit dephosphorylation. After incubation for 15 min on ice, the cells were collected at 3,000*g*, followed by preparation of lysate and western blotting as described above.

RESULTS

Construction of Chimeric Receptors—To analyse the influence of conformation of extracellular and intracellular domains on the signal transduction in EpoR, we developed a series of vectors for chimeric receptors containing a HA-tagged anti-fluorescein ScFv tethered to different combinations of the extracellular D1/D2, TM and the intracellular domains of EpoR (Fig. 1A).

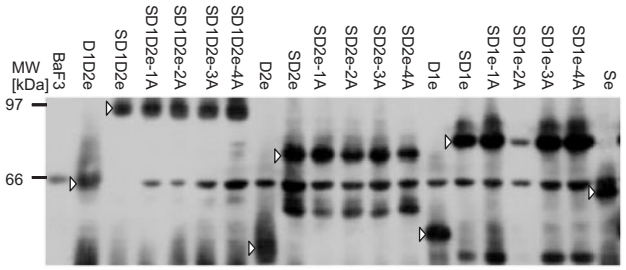

Fig. 2. **Expression of the chimeric receptors.** The EGFP-positive cells transduced with each chimeric receptor were lysed and subjected to western blot analysis using a polyclonal anti-EpoR (C-20) antibody. Parental BaF3 cell lysate was used as a negative control.

First, we fused the HA-tagged ScFv to the extracellular domain of EpoR including both D1 and D2 domains (SD1D2e), or to either the D1 or D2 domain (SD1e or SD2e, respectively). We also removed all of the extracellular D1/D2 domains and directly fused the HA-tagged ScFv to the EpoR TM domain (Se). Constantinescu *et al.* (*14*) reported that periodical changes in the ability of signal transduction were observed when one to four Ala residues were inserted into the cytosolic juxtamembrane region of EpoR. Therefore, we inserted one to four Ala residues after the L^{247} residue in SD1D2e, SD1e and SD2e, to modulate the orientation of the intracellular domain, resulting in SD1D2e-1A to SD1D2e-4A, SD1e-1A to SD1e-4A and SD2e-1A to SD2e-4A, respectively.

Expression of Chimeric Receptors in Ba/F3 pro-B Cells—A murine IL-3-dependent pro-B cell line, Ba/F3, was retrovirally transduced with the expression vector for chimeric receptors. EGFP-positive cells were selected by FACS sorting or by AMEGA (*18*) in the presence of 152 nM BSA-FL. The expression levels of chimeric receptors were analysed by western blotting (Fig. 2). The results confirmed that all of the constructed chimeric receptors were expressed in the transduced cells. The amount of chimeric receptor expressed on the cell surface would influence the signal transduction ability. According to previous reports (*19–23*), most of the nascent EpoRs are retained in the endoplasmic reticulum for degradation, while only a small proportion can exit the endoplasmic reticulum and is expressed on the surface of cells. Therefore, we used a FACS-based assay, in which the HA-tag in each chimeric receptor is stained with mouse anti-HA antibody and PE-labelled secondary antibody to measure the expression level of chimeric receptors on the cell surface (Fig. 3). As a result, all of the chimeric receptors were expressed on the cell surface. However, the levels of expression varied between chimeric receptors. Median fluorescence derived from the PE spanned from 8 (SD1e-3A) to 84 (D1D2e). Interestingly, there was a statistically significant disparity among the chimeric receptors with both D1 and D2 domains (D1D2 chimeras), those with the D1 domain alone (D1 chimeras) and those with the D2 domain alone (D2 chimeras) (Fig. 4). These results suggest that the D2 domain is intrinsically important for the cell-surface expression of the chimeric receptors.

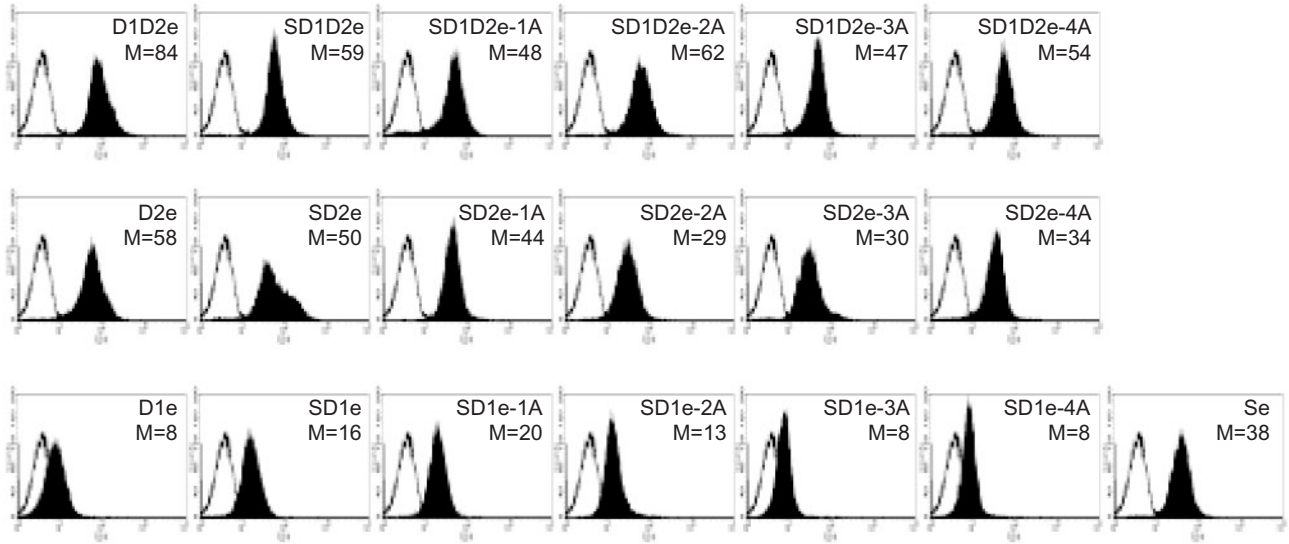

Fig. 3. **Surface expression of chimeric receptors.** The EGFP-positive cells were stained with mouse anti-HA tag antibody followed by PE-labelled secondary antibody (black histogram). A control cell transduced with a chimeric receptor without the HA-tag was used as a negative control (white histogram). Median fluorescence intensities of PE (M) were used to quantify surface expression levels of the chimeric receptors, since this measure of central tendency is least sensitive to outliers.

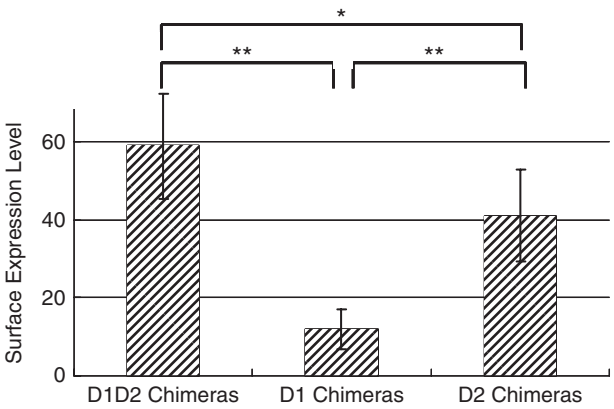

Fig. 4. **Comparison of the surface expression levels of the chimeric receptors with both D1 and D2 domains (D1D2 chimeras), with the D1 domain alone (D1 chimeras) or with the D2 domain alone (D2 chimeras).** The surface expression level of D1D2 chimeras was calculated using the median fluorescence intensities of PE derived from SD1D2e, SD1D2e-1A to SD1D2e-4A. The surface expression levels of D1 chimeras and D2 chimeras were also calculated using the corresponding data. The calculated values are plotted as the mean ± SD $^{**}P < 0.01$; $^{*}0.01 < P < 0.05$.

Cell Proliferation Assay—A cell growth assay was performed to examine whether the cells expressing the chimeric receptors could grow in response to BSA-FL, which could bind to the ScFv domain of the chimeric receptors. This assay is used to characterize the contribution of extracellular and intracellular domains to the signal transduction in the chimeric receptors. Cells were washed and cultured in a series of concentrations of BSA-FL for 3 days, and viable cell concentrations were measured (Fig. 5). As expected, chimeric receptors without ScFv, which were used as negative controls, failed to induce cell growth.

BSA-FL showed agonism at the constructs containing both D1 and D2 domains. SD1D2e succeeded in cell proliferation, but the growth activity was markedly reduced by inserting one to three Ala residues into the intracellular juxtamembrane domain. It was surprising that the growth activity recovered remarkably after insertion of four Ala residues, which also elevated the level of ligand-independent cell proliferation activity relative to that with SD1D2e.

On the other hand, BSA-FL showed inverse agonism at the constructs containing only the D2 domain. The ligand-independent growth induced by SD2e, SD2e-2A and SD2e-3A was inhibited by the addition of BSA-FL. Although SD2e showed strong ligand-independent cell proliferation activity, the insertion of two or three Ala residues weakened the growth activity, which was nearly abolished by the insertion of one or four Ala residues.

Of the constructs that contained only the D1 domain, only SD1e showed growth activity, which was ligand independent. However, the growth activity was abolished by the insertion of Ala residues. BSA-FL showed strong inverse agonism at Se, which contains neither the D1 nor D2 domain.

To confirm the ligand specificity of the BSA-FL-responsive chimeric receptors, we performed a cell proliferation assay using a different set of ligands for the chimeric receptors. Besides BSA-FL, we also tested fluorescein-conjugated OVA (OVA-FL) as a specific ligand, while unconjugated ligands (BSA, OVA and free FL) or specific ligands in the presence of excess free FL (BSA-FL + free FL, OVA-FL + free FL) were tested to investigate the specificity of the chimeric receptors. BSA-FL and OVA-FL contain, on average, two FL molecules on each carrier protein, which would be expected to induce receptor dimerization, a prerequisite for triggering signal transduction. On the other hand, inclusion of excess free FL would be expected to inhibit the signal

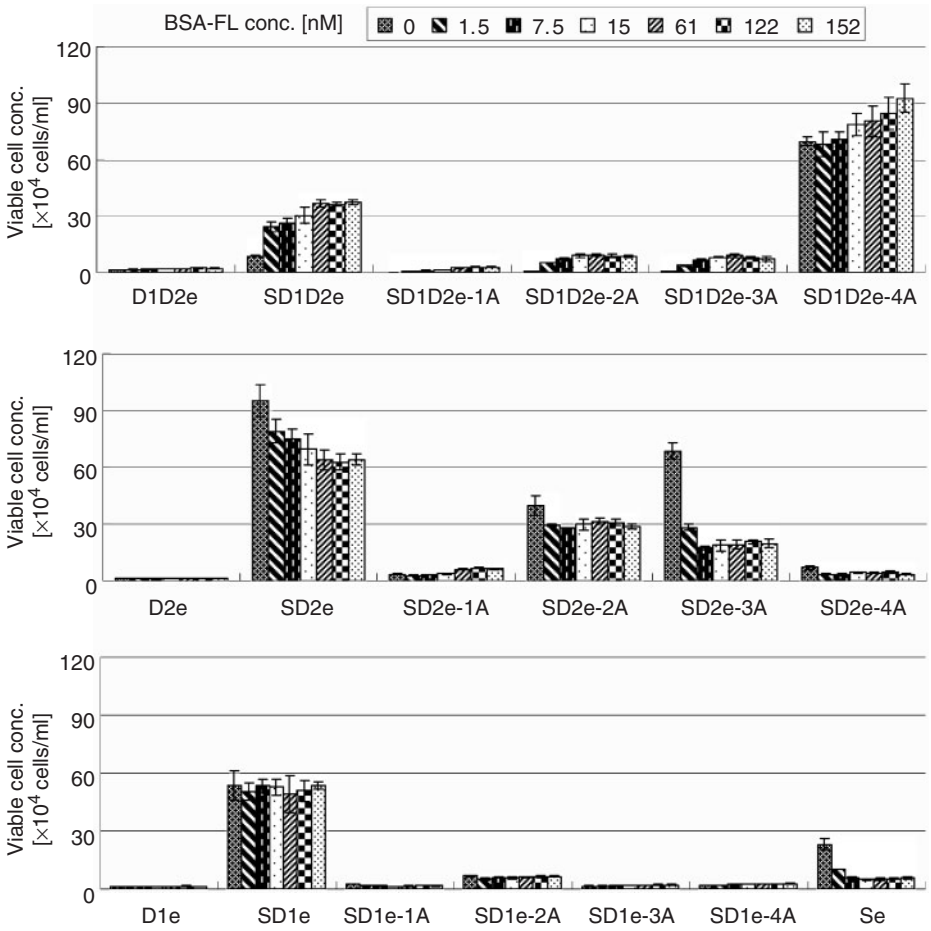

Fig. 5. **Ligand-dependent cell growth induced by chimeric receptors.** The EGFP-positive cells cultured in IL-3 were washed three times with PBS and seeded in 96-well plates at 1,000 cells in 100 μl per well containing various concentrations of BSA-FL. Viable cell concentrations were determined using Cell Counting Kit-8 on day 3. The data from triplicate cultures are plotted as the mean ± SD.

induced by BSA-FL or OVA-FL, since free FL is monomeric and could not induce receptor dimerization. We chose representative chimeric receptors that showed agonism (SD1D2e and SD1D2e-4A), inverse agonism (SD2e, SD2e-3A) and no response (SD1e) to BSA-FL. As expected, all chimeric receptors showed no response to the unconjugated ligands (Fig. 6). The growth responses to OVA-FL were as strong as those to BSA-FL in all of the BSA-FL-responsive transduced cell lines. Furthermore, the cell proliferation in response to BSA-FL or OVA-FL was affected in the presence of excess free FL in all chimeras except the non-responsive SD1e chimera. These results indicate that the chimeric receptors specifically recognize FL molecules on the carrier proteins.

Phosphorylation of Signal Transducers—According to the presently accepted mechanism of signal transduction through EpoR (*2*), dimerization of the intracellular domain of EpoR brings the associated JAK2 molecules into proximity and enables them to transphosphorylate and activate each other and, in turn, phosphorylate the receptor on specific tyrosine residues. Phosphorylated tyrosines provide docking sites for recruitment of SH2 domain-containing proteins, such as SHP1, SHP2, PI-3 kinase, SHIP, Shc, STAT5 and CIS, leading to their phosphorylation and activation of their respective signalling pathways.

To evaluate the influence of extracellular and intracellular domains on the signal transduction in EpoR, the cells expressing the chimeric receptors were stimulated with either IL-3, BSA-FL, Epo, FL dimer-13 (*24*), mouse anti-HA antibody or mouse anti-HA antibody plus anti-mouse IgG. Then, we evaluated the phosphorylation status of signal transducer proteins including JAK2, STAT5 and ERK1/2.

Western blot band intensities (Supplementary Figs 1–3) were determined using ImageJ software, which calculates pixel value statistics of user-defined areas. Phosphorylation levels induced with each ligand were normalized by the respective constitutive expression levels and by levels obtained with IL-3 for each chimeric receptor. The final normalized values were plotted against the rotational angle to see whether Ala insertion at the intracellular juxtamembrane domain induced changes in phosphorylation state (Fig. 7). Since the phosphorylation patterns of ERK1 were similar to those of ERK2, we used data for ERK2 alone. Based on

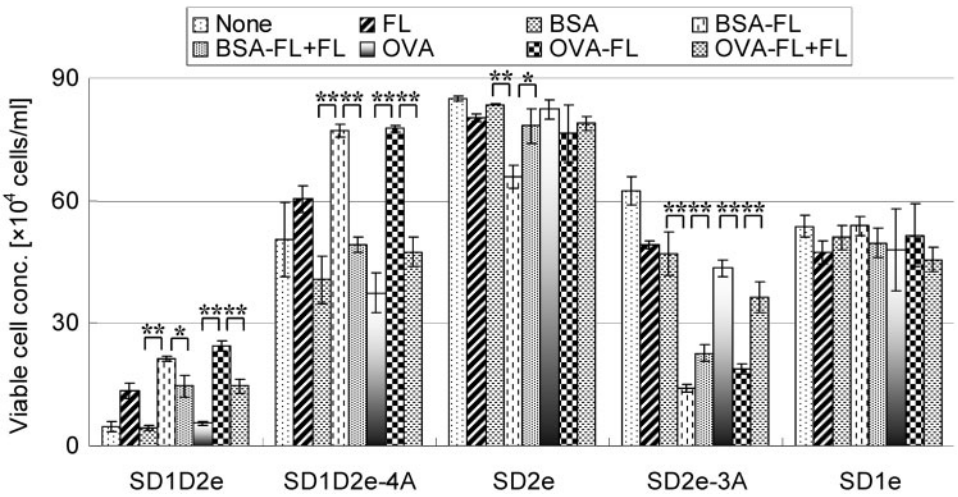

Fig. 6. **Cell growth assay to test ligand specificity.** Representative chimeric receptors that showed agonism (SD1D2e and SD1D2e-4A), inverse agonism (SD2e, SD2e-3A) and no response (SD1e) to BSA-FL were chosen. The EGFP-positive cells cultured in IL-3 were washed three times with PBS and seeded in 96-well plates at 1,000 cells in 100 μl per well without any ligands or with 60 nM BSA, 18 μM free FL, 60 nM BSA-FL, 60 nM BSA-FL with 18 μM free FL, 60 nM OVA, 60 nM OVA-FL or 60 nM OVA-FL with 18 μM free FL. BSA-FL and OVA-FL contain approximately two FL molecules per protein, which was determined from absorbance at 280 nm and 495 nm. Viable cell concentrations of triplicate cultures after 3 days are plotted as the mean ± SD. $^{**}P < 0.01$; $^{*}0.01 < P < 0.05$.

these results, the characteristics of phosphorylation patterns were categorized into the following two points.

(i) In most cases, the phosphorylation statuses of JAK2, STAT5 and ERK2 have a tendency to peak at the intervals of ~220°. The phosphorylation of JAK2, STAT5 and ERK2 showed a peak in SD1D2e, SD2e and SD1e. The phosphorylation levels were reduced after inserting one or three Ala residues, and recovered after insertion of two or four Ala residues. These results suggest that the proliferative signal was strongly affected by the conformation of the intracellular domain of chimeric receptors.

(ii) Different ligands stimulated each chimeric receptor differently. For example, the phosphorylation level of JAK2 in SD1D2e-4A was markedly different to stimulation with either the mouse anti-HA antibody, Epo or BSA-FL. Furthermore, the relative intensity of the phosphorylation level varied among the Ala-inserted mutant receptors. For example, the highest phosphorylation level of JAK2 in SD1D2e-4A was induced with BSA-FL, followed by Epo, mouse anti-HA antibody, mouse anti-HA antibody plus anti-mouse IgG, FL dimer-13 and no stimulation. On the other hand, the highest phosphorylation level of JAK2 in SD1D2e-2A was induced with mouse anti-HA antibody, followed by mouse anti-HA antibody plus anti-mouse IgG, Epo, BSA-FL, FL dimer-13 and no stimulation.

The relative intensity of the phosphorylation level was also dependent on the structure of the extracellular domain. For instance, the highest phosphorylation level of STAT5 in SD1D2e-2A was induced by Epo stimulation, followed by BSA-FL, mouse anti-HA antibody, mouse anti-HA antibody plus anti-mouse IgG, FL dimer-13 and no stimulation. On the other hand, the highest phosphorylation level of STAT5 in SD2e-2A, which has the same intracellular domain as SD1D2e-2A, was induced by mouse anti-HA antibody stimulation, followed by BSA-FL, Epo, no-stimulation, mouse anti-HA antibody plus anti-mouse IgG and FL dimer-13.

Even in the same chimeric receptor, potent ligands for activation of one signal transducer are different from those for activation of the others. For example, the highest phosphorylation level of STAT5 in SD1D2e-2A was induced by Epo, followed by BSA-FL, mouse anti-HA antibody, mouse anti-HA antibody plus anti-mouse IgG, FL dimer-13 and no stimulation. On the other hand, the highest phosphorylation level of ERK2 in the same chimeric receptor was induced by mouse anti-HA antibody plus anti-mouse IgG, followed by Epo, BSA-FL, FL dimer-13 or mouse anti-HA antibody, and no stimulation.

DISCUSSION

In this study, we designed a series of HA-tagged anti-FL ScFv-EpoR chimeras containing the EpoR extracellular D1 or D2 domain alone (D1 chimeras or D2 chimeras, respectively), both D1 and D2 domains (D1D2 chimeras), or no extracellular domains (Se). We also inserted one to four Ala residues into the intracellular juxtamembrane region to alter the orientation of the intracellular domain. These chimeras were individually expressed in the murine pro-B cell line Ba/F3, and we analysed the expression of the chimeras on the cell surface. Consequently, the number of Ala residues inserted at the intracellular juxtamembrane region did not significantly affect the surface expression level (Fig. 3). On the other hand, there was a statistically significant difference in the surface expression level between D1D2 chimeras, D1 chimeras and D2 chimeras (Fig. 4). The cell surface expression of D1D2 chimeras was higher than that of the D1 chimeras as well as the D2 chimeras; in particular, the expression of D1 chimeras was very low.

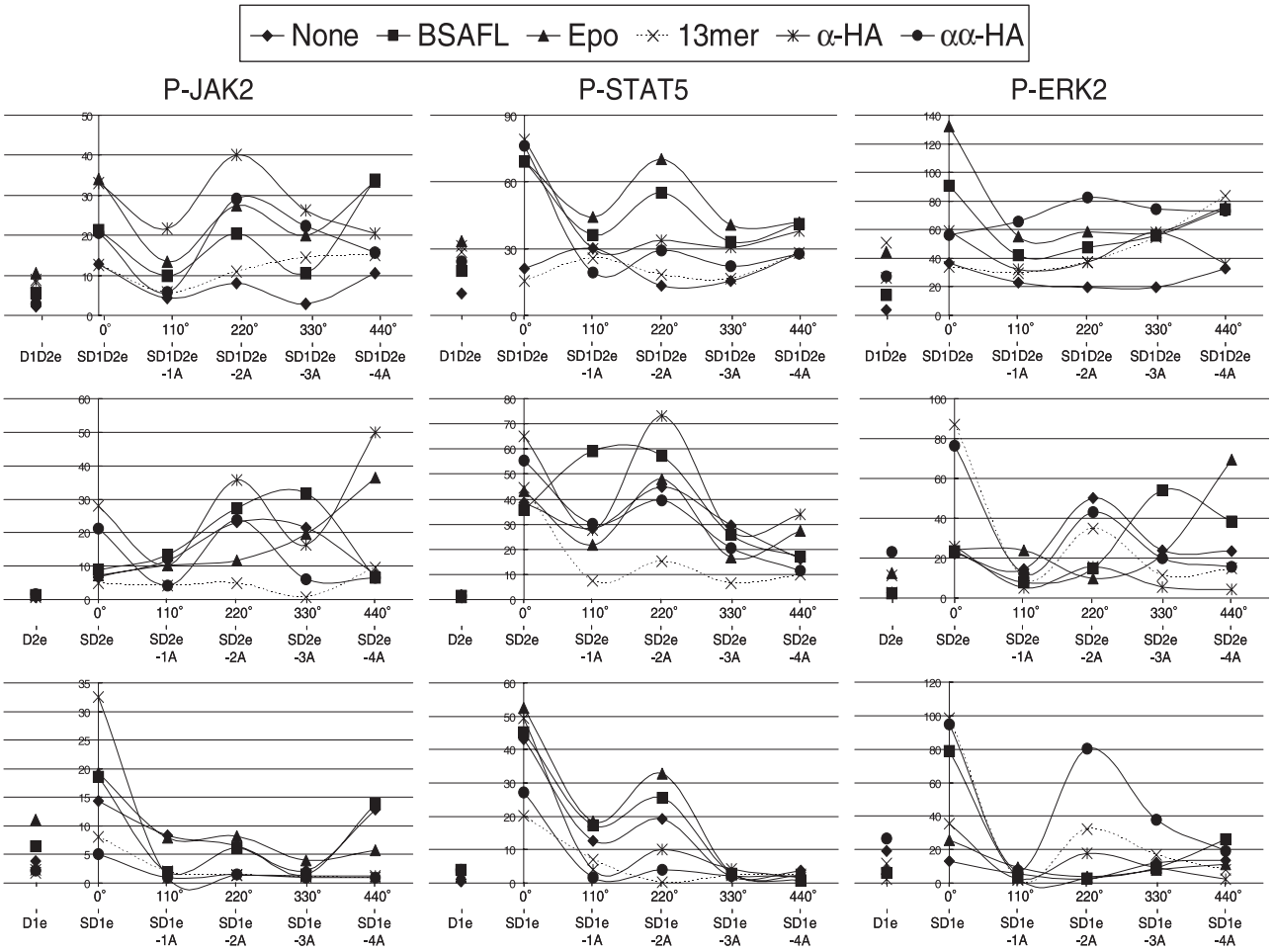

Fig. 7. **Tyrosine or threonine phosphorylation of JAK2, STAT5 and ERK2 induced by chimeric receptors.** The cells expressing each chimeric receptor were stimulated with 67 pM IL-3, 76 nM BSA-FL, 164 pM Epo, 400 nM FL dimer-13 (13-mer), 67 nM mouse anti-HA antibody (α-HA) or 67 nM mouse anti-HA antibody plus 125 nM anti-mouse IgG (αα-HA) for 15 min after the depletion of IL-3 for 12 h. Band intensities were determined using ImageJ software. Phosphorylation levels induced with each ligand were normalized by the respective constitutive expression level and also for levels derived by IL-3 in each chimeric receptor. The final normalized values were plotted against the rotational angle.

Therefore, the domain structure in the extracellular domain is a key determinant for the surface expression level of these chimeras. At the primary sequence level, the extracellular domains, including the four cysteine residues, the spacing of which is conserved, and the five amino acid motif Trp-Ser-Xaa-Trp-Ser (WSXWS) are well conserved and very similar among cytokine receptors. The WSXWS motif or the WSXWS equivalent motif has been shown to be critical for the folding and transport of cytokine receptors to the cell surface (*25*, *26*). In fact, an A234E mutation in the WSAWS motif of EpoR was found to improve the efficiency of the processes (*25*). Since the WSAWS motif is located in the D2 domain of EpoR, the folding and transport of the D1 chimeras to the cell surface might be severely impaired. The difference of the extracellular domain of the chimeras affected not only the surface expression level but also the ligand dependency. BSA-FL showed agonism at the D1D2 chimeras, inverse agonism at the D2 chimeras, and no effect on the D1 chimeras. Furthermore, we stimulated each chimeric receptor with a variety of ligands including BSA-FL, Epo, FL dimer-13 or mouse anti-HA antibody with or without anti-mouse IgG. As a result, different ligands stimulated each chimeric receptor differently. Furthermore, the relative intensity of the phosphorylation level by different ligands was highly dependent on the structure of the extracellular domain. These results suggest that the conformation of the extracellular domain greatly affects the activation of the chimeric receptors.

In addition to the modification of the extracellular domain, the orientation of the intracellular domain in the chimeras was modulated by the insertion of alanine residues. The cell growth assay to examine BSA-FL-dependency revealed that these subtle modifications could greatly affect the signalling activity of the chimeras. In the D1D2 chimeras, the cell proliferation signal was significantly reduced by inserting one to three Ala residues, but was strongly recovered by inserting four Ala residues. In the D2 chimeras, SD2e-2A and SD2e-3A stimulated cell growth, while SD2e-4A and

SD2e-1A failed to stimulate cell growth. The growth signal was lost after inserting the Ala residues in the D1 chimeras. In the stimulation experiments, the phosphorylation levels of JAK2, STAT5 and ERK2 were significantly affected by the number of inserted Ala residues. Such periodical changes of the growth signal is consistent with the previous studies by Constantinescu *et al*. (*14*) and Greiser *et al*. (*15*), who also evaluated alanine-insertion mutagenesis in wild-type EpoR and an IL-5R/gp130 chimera, respectively.

According to the presently accepted mechanism of signal transduction through EpoR, dimerization of EpoR allows the pre-associated JAK2 molecules to trans-phosphorylate and activate each other and, in turn, phosphorylate the receptor on specific tyrosine residues for signal transduction including tyrosines 343, 401, 429, 431, 443, 460, 464 and 479. The phosphorylation of Y479 of EpoR is a key event in the ERK phosphorylation pathway, which recruits the p85 subunit of PI-3 kinase (*27*) and results in activation of ERK2 mitogen-activated protein (MAP) kinase (*28*). On the other hand, STAT5 binds to either phosphorylated Y343 or Y401 of EpoR, and is phosphorylated by the activated JAK2. Therefore, JAK2 is a master enzyme for activation of both STAT5 and ERK2. In the stimulation experiment, the phosphorylation levels of JAK2, STAT5 and ERK2 reached a peak at every two Ala residues (~220°), raising the possibility that JAK2 has two positions per rotation for efficient activation.

In the wild-type EpoR, the extracellular D1 domains exist as a preformed dimer in an open scissor-like conformation in the absence of ligand (*4*), and the oligomerization activity of EpoR TM is found to be very high (*13*). Nevertheless, the ligand-independent cell proliferation is minimized in the wild-type EpoR. On the other hand, the ligand-independent cell proliferation was prominently observed in SD1D2e-4A, SD2e, SD2e-2A, SD2e-3A and SD1e (Fig. 5). This phenomenon would be attributable to the ScFv domain fused to the extracellular N-terminus of each chimeric receptor. Such modification could disturb a switched-off conformational state of unliganded EpoR. This leaky phenotype of chimeric receptors was readily affected by insertion of Ala residues (Fig. 5), indicating the importance of orientation of the intracellular domain for receptor activation. The relatively small ligand dependency of chimeric receptors implies that conformational change from unliganded to liganded receptors would be too small to evoke a dramatic change of the growth activity. It would be an interesting challenge to design a strictly ligand-dependent chimeric receptor.

In summary, here we developed a series of chimeric EpoRs that could be activated by multiple ligands. To our knowledge, this is the first time such an experimental system has been developed to elucidate the mechanism of signal transduction in cytokine receptors. The results of our stimulation experiments indicate that EpoR signal transduction is affected by the conformations of both extracellular and intracellular domains. Therefore, our unique approach using chimeric receptors could contribute to the knowledge of receptor biology, as well as the design of chimeric receptors with altered specificity.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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