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TRYPTOPHAN RESIDUE OF TRP-SER-X-TRP-SER MOTIF IN EXTRACELLULAR DOMAINS OF ERYTHROPOIETIN RECEPTOR IS ESSENTIAL FOR SIGNAL TRANSDUCTION

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SUMMARY: The Trp-Ser-X-Trp-Ser motif commonly exists just outside the transmembrane domains of all cytokine receptors so far isolated. The role of this conserved motif in erythropoietin receptor was examined by assessing a series of mutant receptors on erythropoietin-induced signal transduction. Replacement of one of the two conserved Trp residues in the motif to Gly was found to completely abolish the binding of erythropoietin to the receptor and also to lose the ability to transduce the factor-dependent growth signal. While the mutants with one Ser residue converted to Gly or Ala retained full biological activities, the replacement of both conserved Ser residues diminished the functions of the receptor. Furthermore, the receptors lacking a part or all of the Trp-Ser-X-Trp-Ser motif did not respond to erythropoietin. The Trp-Ser-X-Trp-Ser motif, especially Trp residue, located in extracellular domains of the erythropoietin receptor thus appears to play a critical role in receptor-mediated signal transduction.

Erythropoietin (Epo) is a cytokine which specifically acts on erythroid progenitor cells and stimulates their proliferation and differentiation (1). Structural analysis revealed that the Epo receptor is a member of a large cytokine receptor superfamily, which includes the receptors for IL2, IL3, IL4, IL5, IL6, IL7, granulocyte-macrophage colony-stimulating factor, granulocyte colony stimulating factor, growth hormone, prolactin and leukemia inhibitory factor (2-12). These receptors have common unique structural features: four periodically interspersed cysteines ($CX_{9-10}CXWX_{26-32}CX_{10-15}C$ where C represents cysteine; W, tryptophan and X, a non-conserved amino acid), and Trp-Ser-X-Trp-Ser (WSXWS) motif in the extracellular domains. It is believed that the common cysteine residues hold the conformation of the receptor molecules for their cognate ligand binding. However, the physiological functions of the WSXWS motifs located just outside of the transmembrane domains in all known cytokine receptors have not yet been clarified. In order to understand the role of these unique structural features, we generated a series of mutations in WSXWS motif in mouse Epo receptor and analyzed the biological activities of the mutant Epo receptors on factor-dependent signal transduction.

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Abbreviations: Epo, erythropoietin; IL3, interleukin 3; WSXWS motif, Trp-Ser-X-Trp-Ser motif.

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MATERIALS AND METHODS

Materials and cell lines. Recombinant human Epo (2.2 X 10^5 units/mg) was provided by Kirin Brewery, Tokyo. Recombinant mouse IL3 was prepared from culture supernatant of C127I cells transfected with bovine papilloma vector carrying cDNA for mouse IL3. IL3-dependent mouse pro-B cells LyD9 (13) were maintained in RPMI 1640 medium supplemented with 10 % FCS, 50 μM β-mercaptoethanol and 1 % of culture supernatant of C1271 cells.

Preparations of mutant Epo receptors. The cloned cDNA encoding mouse Epo receptor (7) was inserted downstream of the SRα promoter in the expression vector pME18 containing a neomycine-resistant gene with the simian virus 40 promoter (14), and designated pME-ER. The mutant Epo receptors with single (or double) amino acid replacement in WSXWS motif were constructed using the oligonucleotide-directed *in vitro* mutagenesis system (Amersham). Using single stranded DNA from Epo receptor cDNA subcloned in M13mp18 and the synthetic oligonucleotides [5'-GGCACTCCCGAATCCGCT -3' (ERm1; 232Trp→Gly), 5'-CTCAGACCCGGCACTCC-3' (ERm2; 235Trp→Gly), 5'-AGACCCGGCACTCCCGAATC-3' (ERm3; 232Trp→Gly and 235Trp→Gly), 5'-CCAGG-CACCCCAGAATC-3' (ERm4; 233Trp→Gly), 5'-GGGCTCAGCCCAGGCAC-3' (ERm5; 236Ser→Ala), 5'-GGCTCAGCCCAGGCACCCCAGAAT-3' (ERm6; 233Ser→Gly and 236Ser→Ala)], a series of site directed mutations was introduced. The mutations were confirmed by M13 dideoxynucleotide sequencing, and the mutant Epo receptor cDNAs were inserted into the EcoR I-Xba I site of pME18 expression vector and designated pME-ERm1, m2, m3, m4, m5 and m6, respectively. The deletion mutants in WSXWS motif were prepared by digesting pME-ER with Nhe I, followed by mild digestion with Bal 31 nuclease and blunting with T4 DNA polymerase. After digestion with Xba I, the resultant cDNA fragment was inserted into the Xba I-Acc II site of pME-ER. Sequence analysis revealed that mutants with 18 bp and 39 bp deletions (6 and 13 amino acid deletions) in WSXWS motif had been constructed, and these were designated pME-ERΔ1 and pME-ERΔ2, respectively.

DNA transfection. Plasmid DNAs were transfected into LyD9 cells by electroporation. About 50 μg of Sca I-digested wild type or mutant Epo receptor plasmid DNAs were mixed with 1-2 X 10^7 cells in 800 μl of phosphate-buffered saline containing 11 mM glucose at 4 C, and pulsed once at 500 V and 25 μF and once again at 250 V and 980 μF on ice (Bio-Rad Gene Pulser). Stable transformants resistant to antibiotic G418 (1 mg/ml) were cloned by limiting dilution.

Cell proliferation assay. The proliferation of LyD9 transformants in response to Epo was determined by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)(MTT; Sigma) originally developed by Mosmann (15). Exponentially growing cells (exactly 3 X 10^4) were plated in microtiter plates in 100 μl culture medium in the presence or absence of various concentrations of Epo, and cultured at 37 C for 48 hr. Ten μl of 5 mg/ml MTT in phosphate-buffered saline was added to the cell suspension, and incubated at 37 C for 4 hr. The cell suspension was then mixed thoroughly with 100 μl of 0.04 M HCl in isopropanol. Optical densities were measured on a microplate reader (Bio Rad, model 450), using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Epo binding assay. Recombinant human Epo was labeled using Iodo-Gen (Pierce) and carrier-free ^{125}I in NaOH solution as described (16), and was purified using Sephadex G-25 gel filtration in phosphate-buffered saline containing 0.02 % Tween-20. LyD9 cells (4 X 10^6) grown in the presence of IL3 and the absence of Epo were incubated with various concentrations of ^{125}I-Epo (specific activity, 1.2 X 10^5 Bq/mg) in 300 μl of binding medium (RPMI 1640 medium supplemented with 20 mM HEPES pH 7.4, 1 % bovine serum albumin and 0.4 % sodium azide) at 37 C for 30 min. After sedimentation of the cells through dibutylphthalate oil, cell-associated radioactivities were measured by a gamma counter. Results were corrected for nonspecific binding of ^{125}I-Epo obtained in the presence of 100-fold excess unlabeled Epo and represent specific binding of ^{125}I-Epo.

RESULTS AND DISCUSSION

To explore the physiological function of the WSXWS motif existing in the extracellular domains of the Epo receptor on Epo-induced signal transduction, we generated a series of mutations in WSXWS motif of mouse Epo receptor cDNA using oligonucleotide-directed mutagenesis. The structures of the constructed mutants are summarized in Figure 1. In mutants ERm1 and ERm2, one of the conserved Trp residues (Trp232 and Trp235) is replaced by Gly, and in mutant ERm3 both of the Trp residues are replaced by Gly. Similarly, one of the conserved Ser residues (Ser233 and Ser236) is converted to either Gly or Ala in ERm4 and ERm5 mutants, and both of the Ser residues are converted to Gly and Ala in ERm6 mutant. In addition to these site-directed mutations, two mutants having 6 and 13 amino acid deletions in WSXWS motif are also constructed, and designated ERΔ1 (deletion from Ser233 to Pro238) and ERΔ2 (deletion from Pro226 to Pro238), respectively (Fig. 1). All these mutant Epo receptor cDNAs as well as wild type Epo receptor cDNA were introduced into the pME18 expression vector containing neomycin-resistant gene, and then transfected into IL3-dependent proB cells LyD9. A number of neomycin-resistant clones were established for all mutants, and four each of wild type and mutant transformant clones were further analyzed.

The binding properties of Epo to these stable transformants were first examined. The parental LyD9 cells did not bind Epo (data not shown). The transformant clones which express wild type Epo receptor bound ^{125}I-Epo. Scatchard plot analysis of the Epo binding data revealed that the transformant displayed both high and low affinity receptors (Fig.2A). The dissociation constants (Kd) for high and low affinity are 24 pM and 393 pM, respectively, and the number of receptors for high and low affinity is 160 and 707 per cell,

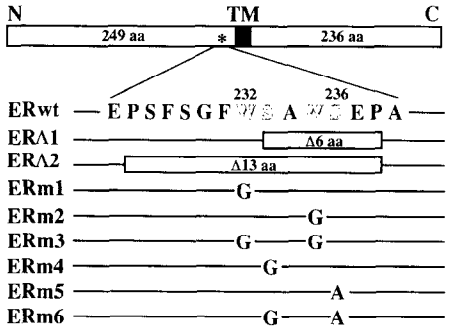

Fig.1. Schematic representation of mouse Epo receptor and the structure of the mutant receptors. Boxes represent the extracellular domains consisting of 249 amino acids and the cytoplasmic domains composed of 236 amino acids. The shaded regions indicate the transmembrane domain. The WSXWS motif is marked by an asterisk, and its wild type sequence is shown on the top line (ERwt). ERΔ1 has a 6 amino acids deletion from Ser233 to Pro238, and ERΔ2 has a 13 amino acids deletion from Pro226 to Pro238. The solid lines indicate the same sequences as wild type receptor. The sequences of site-directed mutants (ERm1-m6) are shown on subsequent lines. G, glycine; A, alanine.

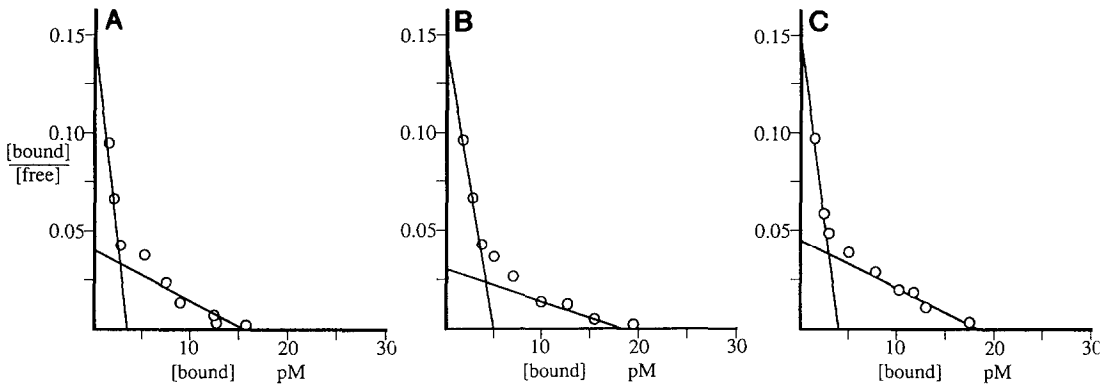

Fig.2. Scatchard plot analysis of ^{125}I-Epo binding to mutant Epo receptors expressed in LyD9 transformants. The wild type Epo receptor cDNA or its mutant cDNAs were transfected into IL3-dependent LyD9 cells by electroporation, and stable transformants were cloned. The transformants (4 X 10^6 in 300 µl of binding medium containing NaN_3) were incubated with various concentrations of ^{125}I-Epo at 37°C for 30 min. Specific binding was determined by subtracting the binding in the presence of 100-fold excess unlabeled Epo. The average of data from three experiments on four independent clones is shown. (A) Cells expressing wild type Epo receptor. (B) Cells expressing ERm4. (C) Cells expressing ERm5.

respectively. These Kd values are consistent with previous results obtained with COS cells (6). Furthermore, the stable mouse L cell transformants expressing Epo receptor have two receptor species (Kd = 26 pM and 300 pM) while the number of receptors per cell is in the order of 10^5 (data not shown). Similarly, the transformants with mutant receptors ERm4 and ERm5, of which one Ser residue in WSXWS motif was replaced by Gly or Ala, bound Epo with similar affinities (Kd for ERm4 = 36 pM and 616 pM; Kd for ERm5 = 28 pM and 463 pM) (Fig.2B and 2C). The number of mutant Epo receptors expressed are in the range 180 to 830 per cell. These values are comparable to those of wild type receptor. On the other hand, no significant Epo binding was observed in LyD9 transformants expressing mutants ERΛ1, ERΛ2, ERm1, ERm2, ERm3 and ERm6, although Northern blot analyses showed that all mutant Epo receptors were transcribed into mRNA in large quantities (data not shown). The expression of these mutants in L cells showed the same results. These observations indicate that either these mutant receptors have lost their Epo binding epitope or they cannot be translocated properly into the cell membrane. Further analyses are required to clarify this point using antibody against Epo receptor. In any case, the replacement of a single Trp (but not Ser) residue in WSXWS motif to another residue abolished completely the physiological function of the receptor, and it is concluded that Trp residue in WSXWS motif is essential for Epo receptor activity.

We next examined the ability of these mutant Epo receptors to transduce growth signal in LyD9 transformants by MTT assay. As shown in Figure 3A, the parental LyD9 cells did not respond to Epo, whereas the transformants expressing wild type Epo receptor responded in a dose-dependent manner. The maximal growth level obtained with Epo was comparable to

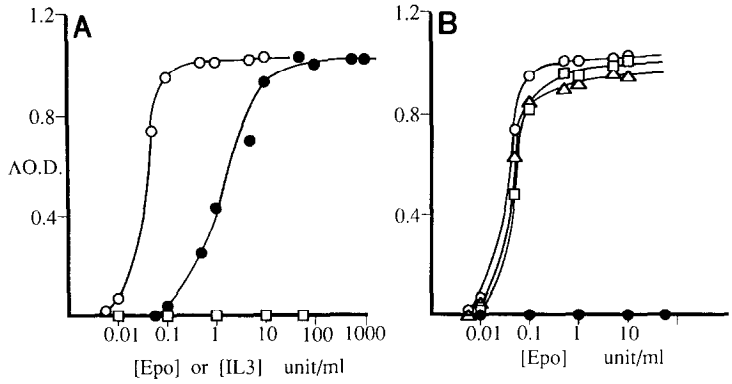

Fig.3. Epo dependent proliferation of LyD9 transformants expressing wild type and mutant Epo receptors. The transformants or the parent cells were cultured in various concentrations of Epo or IL3, and proliferation was quantitated by MTT(tetrazolium) assay. ΔO.D. refers to the optical density (570 nm - 630 nm) of the proliferation assay. Each point is the average of four experiments on four independent clones. (A) Dose dependencies of Epo (open circles) and of IL3 (closed circles) on transformants expressing wild type Epo receptor. Dose dependency of Epo on the parent LyD9 cells (squares). (B) Dose dependencies of Epo on LyD9 cells expressing wild type Epo receptor (open circles) and its mutant ERm4 (triangles), ERm5 (squares) and the other mutants ERΔ1, ERΔ2, ERm1, ERm2, ERm3 and ERm6 (closed circles).

that observed with IL3 (Fig.3A). The transformants expressing mutant receptors having a single Ser residue replacement (ERm4 and ERm5) responded to Epo, although the maximal growth levels of the cells expressing mutant receptors were slightly lower than those of wild type receptor (Fig.3B). The growth signals mediated by the ERm4 and ERm5 mutant receptors were sufficient to sustain long-term cell growth, that is, the transformants expressing ERm4 and ERm5 could be maintained in the medium containing 0.5 unit/ml of Epo instead of IL3. In contrast, none of the transformants with the mutants ERΔ1, ERΔ2, ERm1, ERm2, ERm3 or ERm6 responded to Epo (Fig.3B), nor could any of these transformants survive in the medium containing 2 units/ml of Epo (4 times more than regular concentration) in place of IL3. These results confirmed that both of the conserved Trp (but not Ser) residues in WSXWS motif in the extracellular domains of the Epo receptor are essential for Epo-receptor mediated signal transduction. Very recently, Miyazaki *et al.* (17) reported that the WSXWS motif in IL2 receptor β chain is essential for ligand binding and signal transduction. Our results are consistent with their conclusions, and it could be generalized that every WSXWS motif (especially Trp residue) common to all cytokine receptors has an essential role in the cytokine receptor-mediated signal transduction.

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