A Functional Green Fluorescent Protein-tagged Erythropoietin Receptor Despite Physical Separation of JAK2 Binding Site and Tyrosine Residues*

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Signaling through hematopoietic cytokine receptors such as the erythropoietin receptor (EpoR) depends on the activation of a receptor-bound Janus kinase (JAK) and tyrosine phosphorylation of the cytoplasmic domain. To visualize the EpoR and elucidate structural requirements coordinating signal transduction, we probed the EpoR by inserting the green fluorescent protein (GFP) at various positions. We show that insertion of GFP in proximity to the transmembrane domain, either in the extracellular or the cytoplasmic domain, results in EpoR-GFP receptors incompetent to elicit biological responses in a factor-dependent cell line or in erythroid progenitor cells. Surprisingly, a receptor harboring GFP insertion in the middle of the cytoplasmic domain, and thereby separating the JAK2 binding site from the tyrosine residues, is capable of supporting signal transduction in response to ligand binding. Comparable with the wild type EpoR, but more efficient than a C-terminal EpoR-GFP fusion, this chimeric receptor promotes the maturation of erythroid progenitor cells and is localized in punctated endosome-like structures. We conclude that the extracellular, transmembrane, and membrane-proximal segment of the cytoplasmic domain form a rigid structural entity whose precise orientation is essential for the initiation of signal transduction, whereas the cytoplasmic domain possesses flexibility in adopting an activated conformation.

Ligand binding to membrane-spanning receptors supports signaling networks within cells. The specific structural requirements that enable conversion of ligand binding to the extracellular domain to an activated conformation of the cytoplasmic domain are poorly understood.

Hemopoietic cytokine receptors share common features in the extracellular domain such as four spaced cysteines near the N terminus and a Trp-Ser-X-Trp-Ser (WSXWS) motif located proximal to the cell membrane (for a review, see Refs. 1 and 2). The cytoplasmic domain of hematopoietic cytokine receptors lack intrinsic enzymatic activity and therefore require recruitment of cytoplasmic kinases to promote signal transduction. A simple prototype of the hematopoietic cytokine receptor family is the erythropoietin receptor (EpoR)† that is essential for the development of mature erythrocytes. Crystallographic evidence suggests that in the absence of ligand, the EpoR exists as a preformed dimer in an open scissors-like conformation (3). Upon ligand binding, a conformational switch facilitated by self-interaction of the transmembrane domains is induced, permitting the activation of an intracellular signal transduction cascade (4). This process is supported by a conserved hydrophobic motif localized in the cytoplasmic juxtamembrane domain of the EpoR (5). A continuous stretch of residues in the membrane-proximal domain of the EpoR mediates binding of the Janus kinase JAK2 and ensures transport of the EpoR from the endoplasmic reticulum to the cell surface (6). The precise orientation of critical residues in the juxtamembrane motif is essential for JAK2 activation. Negative inhibitory molecules including the suppressor of cytokine signaling family of proteins (7) and tyrosine phosphatases such as SHP-1 (8), PTP-1B (9), and CD45 (10) tightly regulate JAK2. In addition, JAK2 is involved in activation of signal transducer and activator of transcription protein 1 (STAT1) and STAT3 by the EpoR, as shown by the use of the JAK2 inhibitor AG490 (11). The cytoplasmic domain of the activated EpoR mediates the recruitment of secondary signaling molecules including the lipid kinase phosphoinositide 3-kinase (12, 13) and activation of STATs that promote signal transmission from the cell surface to the nucleus. STAT1, STAT3, and STAT5 are involved in EpoR signal transduction (11, 14–16). Docking of the tyrosine phosphatase SHP-1 leads to termination of signal transduction (8, 17). Signaling pathways activated in response to ligand binding to the EpoR have been studied in detail, but it is unresolved how activation of JAK2 is communicated to phosphorylation of the eight tyrosine residues localized in the membrane-distal cytoplasmic domain.

Here we present a set of EpoR-GFP fusion proteins that are 1) ER-retained and signaling-incompetent, 2) surface-expressed but signaling-incompetent, and 3) surface-expressed and signaling-competent. Our analysis shows that the cytoplasmic domain of the EpoR can tolerate a large insertion separating the JAK2-activating segment from the respective tyrosine residues and yet coordinate biological responses supporting proliferation and differentiation of erythroid progenitor cells.

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1 The abbreviations used are: EpoR, Epo receptor; STAT, signal transducers and activators of transcription; GFP, green fluorescent protein; HA, hemagglutinin; TM, transmembrane; CFU-E, colony-forming unit-erythroid; ER, endoplasmic reticulum.
**EXPERIMENTAL PROCEDURES**

**Constructs—**Primers used are summarized in Table I. Thermostabilized amino acid exchanges V163A, I167T, and S175G were introduced into the cDNA of enhanced green fluorescent protein (CLONTECH, Palo Alto, CA) by overlap extension PCR using as general 5’-primer primer numbers 1 and introduces a BglII restriction site and as general 3’-primer number 2 encoding an EcoRI restriction site. V163A and I167T were introduced concomitantly using the primers 3-U and 3-L. Using the resulting cDNA as a template, S175G was introduced with the wild type EpoR or EpoR-GFP chimera were analyzed by saturation binding of 125I-labeled Epo as described (20). Surface expression of HA-tagged EpoR was evaluated by flow cytometry (FACScan; Becton Dickinson, Palo Alto, CA) and analyzed for green and red fluorescence by flow cytometry.

**Immunoprecipitation and Immunoblotting—**BaF3 cells expressing the wild type EpoR or EpoR-GFP chimera were selected in puromycin and stained with an anti-HA (Roche Molecular Biochemicals) primary antibody and anti-STAT5b (Upstate Biotechnology) secondary antibody and analyzed by fluorography.

**Antibodies—**Western blots were performed using anti-epo receptor (Santa Cruz Biotechnology), anti-p85 subunit of phosphatidylinositol 3-kinase (kindly provided by Dr. Lewis Cantley, Harvard Medical School, Boston, MA), and anti-SHP1 (Santa Cruz Biotechnology) antisera. The blots were stripped and reprobed with anti-EpoR, anti-STAT5b, anti-JAK2, anti-SHIP-1 (all purchased from Santa Cruz Biotechnology), and anti-p85 subunit of phosphatidylinositol 3-kinase antisera (kindly provided by Dr. Lewis Cantley).
day 13.5 Balb/c were grown on coverslips precoated with 0.2% gelatin (Sigma) for 20 h in Iscove’s modified Eagle’s medium, 30% fetal calf serum supplemented with 0.5 unit/ml Epo. The cells were washed and analyzed with a Leica DM IRE2 confocal microscope.

RESULTS

GFP Insertions in the Erythropoietin Receptor—To visualize the EpoR and to check whether a visible EpoR is capable of activation of signal transduction in response to ligand binding, we inserted the GFP at four positions of the EpoR (Fig. 1). In the resulting chimeric proteins, GFP is either located at the junction between the extracellular and TM domains (EpoR-GFP1) or at various positions within the cytoplasmic domain. In EpoR-GFP2, the insertion of GFP directly after the TM domain alters the spacing between the hydrophobic juxtamembrane motif and the JAK2 binding sites, whereas in EpoR-GFP3 the JAK2-activating domain is separated from the eight cytosolic tyrosine residues that mediate the recruitment of signaling molecules. The least invasive chimeric receptor is EpoR-GFP4, where GFP is fused to the C terminus of the EpoR.

GFP Insertion in the Cytoplasmic Domain Does Not Impair Cell Surface Expression of the EpoR—To test the functionality of the EpoR-GFP receptors, wild type EpoR and chimeric receptors were stably expressed in the interleukin-3-dependent pro-B cell line BaF3. Analysis of total cell lysates by immunoblotting with anti-EpoR antiserum revealed that EpoR-GFP1, EpoR-GFP2, and EpoR-GFP3 were expressed at levels comparable with wild type EpoR, whereas EpoR-GFP4 reproducibly showed reduced expression levels (Fig. 2A). To evaluate whether GFP insertion affected surface transport of the chimeric receptors, we measured \(^{125}\text{I}-\text{Epo binding to BaF3 cells stable expressing the EpoR derivatives. As shown in Fig. 2B, chimeric receptors harboring the GFP insertion in the cytoplasmic domain bound the ligand to a similar degree as wild type EpoR. It should be noted that Epo binding to EpoR-GFP2 was reproducibly enhanced. However, EpoR-GFP1 that contains GFP in the extracellular domain did not show significant Epo binding. To distinguish whether the lack of Epo binding was caused by the inability to engage the ligand or by impaired cell surface expression, we introduced an HA tag in the extracellular domain of EpoR-GFP1, EpoR-GFP2, and wild type EpoR. Flow cytometry analysis of BaF3 cells stable expressing the HA-tagged receptors showed that whereas wild type EpoR and EpoR-GFP2 were detected on the cell surface, EpoR-GFP1 was below the detection limit (Fig. 2C). This suggests that GFP insertion in the extracellular domain of the EpoR blocks transport to the cell surface, whereas insertion at various positions of the cytoplasmic domain does not impair cell surface prevalence.

A Chimeric Receptor Containing GFP in the Middle of the Cytoplasmic Domain Successfully Coordinates Signaling and Biological Responses—To elucidate whether GFP insertion in
the cytoplasmic domain of the EpoR affected the activation of signal transduction. Epo-induced signaling was studied in BaF3 cells expressing wild type EpoR or the EpoR-GFP chimera. Tyrosine-phosphorylated JAK2 and EpoR were measured as indicators for Epo-mediated signal transduction (Fig. 3A). Detergent lysates of cells left untreated or stimulated with Epo were subjected to immunoprecipitation with anti-EpoR or anti-JAK2 antiserum and subsequently analyzed by immunoblotting with an anti-phosphotyrosine monoclonal antibody. As expected, ligand addition to cells expressing wild type receptor resulted in efficient tyrosine phosphorylation of the EpoR and JAK2. A receptor chimera that is not transported to the cell surface (EpoR-GFP1) was unable to trigger tyrosine phosphorylation of the receptor or JAK2. However, despite its presence on the cell surface, EpoR-GFP2 was not able to activate signal transduction, suggesting that structural continuity of the hydrophobic juxtamembrane domain motif and the JAK2 binding sites is required for efficient signal conversion. The chimeric receptor EpoR-GFP4 was tyrosine-phosphorylated upon Epo addition, albeit to a lower extent than wild type EpoR. This may be due to the reduced expression of this receptor variant. Surprisingly, a receptor chimera containing the GFP insertion in the middle of the cytoplasmic domain (EpoR-GFP3) mediated JAK2 and EpoR tyrosine phosphorylation, indicating that the cytosolic domain of the EpoR is capable of coordinating JAK2 activation and receptor tyrosine phosphorylation despite physical separation by GFP insertion. Whereas the unphosphorylated forms of EpoR-GFP3 and EpoR-GFP4 showed comparable mobility, indicating that GFP insertion had no major effect, the tyrosine-phosphorylated form of EpoR-GFP3 showed higher mobility. Therefore, we asked whether this is caused by partial tyrosine phosphorylation of EpoR-GFP3. The phosphorylation of critical tyrosine residues in EpoR-GFP3 compared with EpoR-GFP4 was determined by their capacity to bind the Src homology 2 domain-containing signaling molecules STAT5, SHP1, and p85 (Fig. 3B). As evidenced by immunoprecipitation experiments from detergent lysates of cells that were left either unstimulated or treated with Epo, both the tyrosine-phosphorylated forms of EpoR-GFP3 and EpoR-GFP4 were able to associate with STAT5, SHP1, and p85 comparable with wild type EpoR. Therefore, both receptor chimeras are indistinguishable regarding their capacity to recruit signaling molecules. It is possible that underphosphorylation of one of the tyrosine residues to which binding partners have not yet been identified accounts for the difference in electrophoretic mobility.

To test whether the initiation of signal transduction mediated by the chimeric receptors resulted in efficient biological responses, we first tested the capacity of the EpoR-GFP receptors to support the growth of BaF3 cells in the presence of Epo. BaF3 cells expressing either wild type EpoR or various EpoR-GFP chimeras were cultured in the presence of increasing concentrations of Epo ranging from 0.1 to 10 Epo units/ml for 3 days. The cell numbers shown in Fig. 4A indicate that EpoR-GFP3 supported cell proliferation to a similar extent as wild type EpoR, in particular at low Epo concentration, whereas EpoR-GFP4 showed reduced capacity in promoting proliferation. Confirming the biochemical analysis, EpoR-GFP chimera that did not activate signal transduction was unable to support proliferation of BaF3 cells in the presence of Epo. To further
test the biological function of the EpoR-GFP chimera, the receptors were introduced into fetal liver cells of EpoR−/− mice by retroviral transduction and tested for their ability to support the formation of CFU-E colonies in the presence of Epo. In agreement with the cell proliferation experiments, EpoR-GFP3 supported similar numbers of CFU-E colonies compared with wild type EpoR, whereas EpoR-GFP4 reproducibly resulted in a lower number of CFU-E colonies. Again, EpoR-GFP1 and EpoR-GFP2 were unable to promote proliferation and terminal differentiation of erythroid progenitor cells. Thus, unexpectedly, an EpoR-GFP receptor containing GFP inserted in the middle of the cytoplasmic domain was functionally indistinguishable from the wild type EpoR, whereas direct fusion of GFP to the C terminus of the EpoR resulted in a receptor with reduced activity.

**In Vivo Localization of the EpoR**—To test whether the EpoR-GFP chimeras are detectable by fluorescence microscopy and whether the fluorescence intensity is sufficient to monitor EpoR trafficking in living cells, we analyzed the chimeric receptors expressed in transiently transfected 293T cells (A) and in transduced fetal liver cells (B). The cells were fixed with paraformaldehyde and permeabilized with 0.2% Triton X-100. HA-EpoR was detected with a rat antibody raised against HA and a secondary anti-rat IgG coupled to Alexa594. Transfection efficiencies were comparable for all constructs. Confocal images were taken 48 h after transient transfection using a Leica DM IRE2 confocal microscope.

**Fig. 4.** EpoR-GFP chimeric receptors capable of signal transduction support proliferation and differentiation. A, proliferation of parental BaF3 cells or BaF3 cells expressing the EpoR or EpoR-GFP chimera in response to Epo. Cell numbers were determined using a Coulter counter. Growth is displayed as the mean percentage ± S.D. of the cell numbers obtained in WEHI conditioned medium for three independent cell pools. The experiment was performed four times with similar results. B, formation of CFU-E colonies upon expression of the EpoR or EpoR-GFP chimera in fetal liver cells from EpoR−/− mice. Transduced fetal liver cells from EpoR−/− mice were plated in methylcellulose supplemented with 4 units/ml Epo. The values plotted (mean ± S.D., n = 3) represent the number of CFU-E colonies that were counted upon benzidine staining of hemoglobinized cells. Similar results were obtained in three independent experiments. Comparable gene transfer rates of the transducing supernatants were confirmed by measuring GFP expression in transduced wild type fetal liver cells by flow cytometry.

**Fig. 5.** The EpoR localized to endosomal structures in living cells. Confocal microscopy of GFP-EpoR and HA-EpoR in transfected 293T cells (A) and in transduced fetal liver cells (B). The cells were fixed with paraformaldehyde and permeabilized with 0.2% Triton X-100. HA-EpoR was detected with a rat antibody raised against HA and a secondary anti-rat IgG coupled to Alexa594. Transfection efficiencies were comparable for all constructs. Confocal images were taken 48 h after transient transfection using a Leica DM IRE2 confocal microscope.

**DISCUSSION**

Signal conversion through cytokine receptors relies on intricate communication between the extracellular ligand binding domain and the cytosolic domain that mediates recruitment of signaling molecules. Here, we demonstrate that signal transduction via the EpoR, a member of the cytokine receptor superfamily, can occur despite physical separation of the JAK2 binding site from the cytosolic tyrosine residues, which are phosphorylated upon stimulation with Epo.

Insertion of GFP at the junction between the extracellular and TM domains results in a chimeric receptor not transported to the cell surface and unable to bind ligand. This phenotype is reminiscent of mutations in the WSXWS motif of the EpoR extracellular domain, since deletion or alterations in the WSXWS motif resulted in receptors that were retained in the ER and unable to interact with the ligand (22). The WSXWS motif is conserved in the extracellular domain of cytokine receptors and was initially believed to be involved in ligand binding. However, the crystal structures of the extracellular domain of the growth hormone receptor (23) and the EpoR (24) showed that the WSXWS motif is located away from the interfaces that bind the respective ligand. The phenotype of the WSXWS mutants rather suggested that the intact motif is necessary for correct trafficking of the receptor. Our results indicate that not only the amino acid sequence of the motif but...
also the spatial localization in close proximity to the cell membrane could be critical for successful transport of the EpoR to the cell surface.

Recent evidence suggests that JAK2 recruitment to the EpoR mediated by a continuous block of residues in the membrane-proximal segment of the cytoplasmic domain is required for EpoR cell surface expression (6). Our analysis of EpoR-GFP2 shows that increasing the distance between the JAK2 binding motif in the cytoplasmic part of the EpoR and the cell membrane does not disturb the surface prevalence of the EpoR. However, physical separation of the JAK2 binding sites from the precisely oriented hydrophobic motif in the juxtamembrane segment (5) abrogates the activation of signal transduction. This suggests that the ligand binding domain, the TM domain, the membrane-proximal hydrophobic patch, and the JAK2 binding sites are organized in a structurally rigid entity that requires precise spatial alignment to activate signal transduction.

The major part of the cytosolic domain encompassing box 2 and the eight tyrosine residues is contained in exon 8 of the EpoR genomic locus, suggesting a conserved functional entity. Yet we show that insertion of GFP in the middle of the cytoplasmic domain results in a chimeric receptor (EpoR-GFP3) capable of initiating signal transduction and biological responses comparable with wild type EpoR. The cytoplasmic domain of the EpoR is partially unfolded in the absence of JAK2 (25), indicating that JAK2 acts as a molecular chaperone (6) and is required for structural organization of the cytoplasmic domain. Our results demonstrate that tyrosine phosphorylation of the cytoplasmic domain is maintained despite physical separation of the JAK2-activating domain from the segment harboring the tyrosine residues. This suggests that in the activated state, JAK2 possesses flexibility in accessing substrate tyrosine residues and/or that additional JAK2 coordination sites exist in the membrane-distal segment of the EpoR cytoplasmic domain (6). The possibility that another kinase can compensate for JAK2 is rather unlikely, since JAK2 null mice show a dramatic phenotype with fetal anemia and embryonic lethality at day 12.5 comparable with the EpoR null mice (26, 27).

Previous studies in other receptor systems have been limited to the analysis of C-terminally GFP-tagged receptors (28, 29). However, the EpoR that contains GFP fused to the C terminus (EpoR-GFP4) is expressed at reduced levels and has a decreased capacity to promote the formation of CFU-E colonies. This in chimeric protein, GFP is localized in close proximity to Tyr479, a residue that has been shown to be important for the recruitment of the lipid kinase phosphoinositide 3-kinase (12, 13) and sufficient in the absence of other tyrosine residues to promote the biological functions of the EpoR (12, 30). Indeed, further separation of Tyr479 and GFP improved signal transmission and the capacity to support the biological functions, although the overall expression levels remained reduced (2).

In summary, we show by marking a hematopoietic cytokine receptor with a GFP insertion that the extracellular, transmembrane, and membrane-proximal domains form a rigid structure whose specific orientation is essential for initiating signal transduction in response to ligand binding. However, we propose that additional coordinating mechanisms exist, since long range activation of the membrane-distal part is possible, providing a novel concept how ligand binding is converted to receptor activation.

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