THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 29, Issue of July 19, pp. 26547–26552, 2002
Printed in U.S.A.


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

Received for publication, March 8, 2002, and in revised form, May 2, 2002
Published, JBC Papers in Press, May 7, 2002, DOI 10.1074/jbc.M202287200

**Robin Ketteler‡§, Achim C. Heinrich‡¶, Julia K. Offe‡, Verena Becker‡, Jacob Cohen‖, Drorit Neumann‖**, and Ursula Klingmüller‡§**‡‡**

*From the ‡Max-Planck-Institute of Immunobiology, 79108 Freiburg, Germany and ‖Sackler-Faculty of Medicine, Department of Cell Biology and Histology, Tel-Aviv University, 69978 Ramat-Aviv, Israel*

**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.

Hematopoietic 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 (WS*X*WS) 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)[1] 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.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by Sonderforschungsbereich SFB364.

¶ Supported by the Boehringer Ingelheim Fonds.

** Supported by German Israeli Foundation Grant I-666-79.2/2000.

‡‡ To whom correspondence should be addressed: Max-Planck-Institute for Immunobiology, Stübeweg 51, 79108 Freiburg, Germany. Fax: 49-761-5108-358; E-mail: klingmueller@immunbio.mpg.de.

[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.

TABLE I
*Primers used for construction of EpoR-GFP chimeras*

| Primer # | sequence |
|---|---|
| #1 | 5'-GCA TCA GAT CTA CCA TGG TGA GCA A-3' |
| #2 | 5'-GCA TCG AAT TCT TAG GAT CCC TTG TAC AGC TCG T-3' |
| #3-U | 5'-AGA AGA ACG GCA TCA AGG CCA ACT TCA AGA CCC GCC ACA ACA TCG-3' |
| #3-L | 5'-TCG ATG TTG TGG CGG GTC TTG AAG TTG GCC TTG ATG CCG TTC TTC-3'. |
| #4-U | 5'-CAT CGA GGA CGG CGG CGT GCA GCT CG-3' |
| #4-L | 5'-CGA GCT GCA CGC CGC CGT CCT CGA TG-3' |
| #5 | 5'-GCATGATCAGTCGACACCATGGACAAACTCAG-3' |
| #6-U | 5'-GCTAGCGACCTGGATCCTCTCATCTTGACG-3' |
| #6-L | 5'-CGTCAAGATGAGAGGATCCAGGTCGCTAGC-3' |
| #7 | 5'-GCTGAATTCTCAGATCTTCTGCTG-3' |
| #8-L | 5'-CCCTTGCTCACCATGTCCAGGTCGCTAG-3' |
| #8-U | 5'-CTAGCGACCTGGACATGGTGAGCAAGGG-3' |
| #9 | 5'-AGAGGATCCAGGTCGCTAGCCTTGTACAGCTCGTC-3' |
| #10 | 5'-GTCGAATTCCTAGGCGGCCGCAGGTGGATCCTC3' |
| #11 | 5'-CGAAGATCTGGATGGTGAGCAAGGGC-3' |
| #12 | 5'-GTCGGCGGCCGCCTTGTACAGCTCGTC-3' |
| #13 | 5'-GACGCGGCCGCCAAGATCTGGCCTGG-3' |
| #14 | 5'-GTCGAATTCCTAGGAGCAGGCCA-3' |
| #15 | 5'-CTGCAGCAGAAGATCTGGCC-3' |
| #16-L | 5'-GCCCTTGCTCACCATGCTACTGCCCACCGGC-3' |
| #16-U | 5'-GCCGGTGGGCAGTAGCATGGTGAGCAAGGGC-3' |
| #17 | 5'-GTCGGCATGCTCGGATCCCTTGTACAGCTCGTC-3' |
| #18 | 5'-GTCGAATTCCTAGGCGGCCGCGGAGCAGGCCACA-3' |
| #19 | 5'-GCACCGTTGGCGGCCGCCACCATGGTGAGCAAGGG-3' |
| #20 | 5'-GCATCGAATTCTTAGGATCCCTTGTACAGCTCGT-3' |

## EXPERIMENTAL PROCEDURES

*Constructs*—Primers used are summarized in Table I. Thermostabilizing 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 number 1 that introduces a *Bgl*II restriction site and as general 3′-primer primer 2 encoding an *Eco*RI 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 primers 4-U and 4-L. The EpoR-GFP receptors were generated by overlap extension PCR including the following steps. To generate EpoR-GFP1, first a shortened EpoR was established harboring a *Bam*HI restriction site at amino acid position 224 in the EpoR without altering the amino acid sequence. The shortened EpoR fragment flanked by *Bcl*I/*Sal*I (5′) and *Bgl*II/*Eco*RI (3′) restriction sites was generated using primers 5, 6-L, 6-U, and 7 and was cloned into the *Bam*HI and *Eco*RI restriction sites in the retroviral vector pBABE (pBABE-EpoR-*Bam*HI). Second, an in-frame fusion of the EpoR extracellular domain and GFP was established using primers 5, 8-L, 8-U, and 9. The joined fragment was subcloned via the *Sal*I and *Bam*HI in pBABE-EpoR-*Bam*HI. Finally, inserting the EpoR *Bgl*II/*Eco*RI restriction fragment into pBABE-EpoR-*Bam*HI, resulting in pBABE-EpoR-GFP1, completed the EpoR cDNA.

To generate EpoR-GFP2, an EpoR subfragment (amino acids 1–304) encompassing the EpoR extracellular domain and transmembrane (TM) domain was produced using primers 5 and 10. The PCR fragment was digested with *Bcl*I and cloned into pBABE cut with *Bam*HI and *Sal*I (blunt), resulting in pBABE-EpoR-*Not*I. By PCR amplification, *Bgl*II (5′) and *Not*I (3′) restriction sites were introduced at the respective ends of the GFP cDNA using primers 11 and 12. The PCR fragment was subcloned via *Bgl*II and *Not*I in pBABE-*Not*I. The EpoR cDNA was completed by PCR amplification using primers 13 and 14 and inserting the PCR fragment *Not*I and *Eco*RI in pBABE-EpoR-*Not*I, resulting in pBABE-EpoR-GFP2. EpoR-GFP3 bearing GFP inserted at amino acid position 336 in the EpoR cDNA was generated by using primers 15, 16-L, 16-U, and 17 and subcloned via *Bgl*II and *Sph*I into the respective restriction sites in pBS-EpoR that harbors the EpoR cDNA inserted into *Bam*HI and *Eco*RI in pBluescript II KS (Stratagene).

EpoR-GFP4 harbors GFP fused to the C terminus of the EpoR and was generated by introducing *Not*I and *Eco*RI restriction sites at the 3′-end of the EpoR cDNA. The EpoR cytoplasmic domain was amplified using primers 13 and 18 and subcloned via *Bam*HI and *Eco*RI into pBABE-EpoR-*Not*I, resulting in pBABE-EpoR-*Not*Icyto. *Not*I and *Eco*RI restriction sites flanking the GFP cDNA were amplified by PCR with primers 19 and 20 and inserted via *Not*I and *Eco*RI in pBABE-EpoR-*Not*Icyto.

The resulting EpoR-GFP cDNAs were verified by automated sequencing and inserted via *Sal*I and *Eco*RI digestion into pMOWS (18).

The HA tag was inserted into wild type EpoR by excising the HA-containing fragment with *Eco*RI and *Bam*HI from pMX-HA-EpoR-IRES-GFP (kindly provided by Dr. Stefan Constantinescu, Ludwig Institute for Cancer Research, Brussels, Belgium) and subcloning it into the *Eco*RI and *Pac*I restriction sites of pMOWS-EpoR, yielding pMOWS-HA-EpoR. HA-EpoR-GFP1 and HA-EpoR-GFP2 were generated by subcloning the *Pml*I and *Bam*HI fragment from pMOWS-EpoR-GFP1 or pMOWS-EpoR-GFP2 into the corresponding sites of pMOWS-HA-EpoR.

*Cell Lines and Cultures*—The retroviral vectors were transiently transfected in Phoenix-Eco cells by calcium phosphate precipitation (18) and visualized after 24 h or used for the production of transducing supernatants as described (18). Transducing supernatants were applied to introduce the cDNA for the EpoR or EpoR-GFP chimera into the interleukin-3-dependent pro-B cell line BaF3 and fetal liver cells.

Pools of BaF3 cells expressing the wild type EpoR or the EpoR-GFP chimera were selected in 1.5 $\mu$g/ml puromycin (Sigma) 48 h after transduction. Cell pools expressing comparable amounts of the receptors were identified by immunoblotting and used for further experiments. The selected cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 10% WEHI-conditioned medium in the presence of 1.5 $\mu$g/ml puromycin.

Fetal liver cells derived from 12.5-day-old embryos from EpoR$^{-/-}$ mice (19) were prepared and transduced as described (18). The transduced cells were plated in 0.8% methylcellulose (StemCell Technologies, Vancouver, Canada) supplemented with 4 units/ml Epo (Cilag-Jansen, Bad Homburg, Germany). Colony-forming unit-erythroid (CFU-E) colony formation was monitored by benzidine staining of hemoglobinized cells. To ensure comparable transduction rates, GFP expression was assessed by fluorescence-activated cell sorting analysis (FACScan; Becton Dickinson, Palo Alto, CA) in transduced fetal liver cells of wild type mice after 20 h of cultivation in Iscove's modified Eagle's medium supplemented with Epo.

*Surface Binding*—Three independent pools of BaF3 cells expressing the wild type EpoR or EpoR-GFP chimera were analyzed by saturation binding of $^{125}$I-labeled Epo as described (20). Surface expression of HA-tagged EpoR was evaluated by flow cytometry (FACScan). BaF3 cells selected in puromycin were incubated with rat anti-HA (Roche Molecular Biochemicals) as primary antibody and anti-rat IgG coupled to Cy5 (Dianova, Hamburg, Germany) as secondary antibody and analyzed for green and red fluorescence by flow cytometry.

*Immunoprecipitation and Immunoblotting*—BaF3 cells expressing the wild type EpoR or the EpoR-GFP chimera were starved for 3 h in RPMI with 1 mg/ml bovine serum albumin and then stimulated for 5 min at 37 °C with 50 units/ml Epo. Detergent lysates equivalent to $1 \times 10^7$ cells were prepared using Nonidet P-40 buffer as described (8) and subjected to immunoprecipitation using anti-EpoR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-JAK2 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-STAT5b (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) antiserum. The immunoprecipitates were eluted, resolved by 15% SDS-PAGE, and transferred to a nitrocellulose membrane. Detection by immunoblotting was performed with an anti-phosphotyrosine monoclonal antibody (4G10, Upstate Biotechnology, Inc., Lake Placid, NY) followed by enhanced chemoluminescence (Amersham Biosciences). The blots were stripped and reprobed with anti-EpoR, anti-STAT5b, anti-JAK2, anti-SHP-1 (all purchased from Santa Cruz Biotechnology), and the anti-p85 subunit of phosphatidylinositol 3-kinase antiserum (kindly provided by Dr. Lewis Cantley).

*Growth Assay*—BaF3 cells expressing the wild type EpoR or the EpoR-GFP chimera were washed three times with RPMI and plated at a density of $5 \times 10^4$ cells/well in 24-well plates in the presence of Epo concentrations ranging from 0.1 to 10 units/ml or 10% WEHI conditioned medium as a source for interleukin-3.

After 3 days, cell numbers were determined using a Coulter counter and expressed as the percentage of growth obtained in a parallel well containing 10% WEHI conditioned medium instead of Epo.

*Confocal Microscopy*—The localization of GFP-EpoR fusion proteins and HA-tagged EpoR was assessed in 293T cells transiently transfected with retroviral expression vector constructs. The cells were grown on coverslips in six-well plates and either directly analyzed by immunofluorescence or fixed with 3% *para*-formaldehyde for 15 min at room temperature prior to immunostaining. For co-staining of HA-tagged receptors, the cells were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. After three washes in phosphate-buffered saline, the cells were incubated with an antibody raised against HA (Roche Molecular Biochemicals). After three washes, the cells were incubated with an anti-rat IgG coupled to Alexa594 (Molecular Probes, Inc., Eugene, OR). All incubations with antibodies were performed at 4 °C in phosphate-buffered saline supplemented with 0.3% bovine serum albumin. The antibodies were used as 1:100 dilutions. Fetal liver cells from

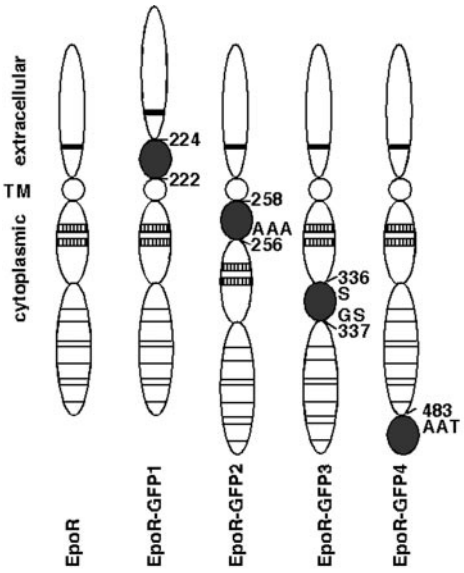


FIG. 1. **EpoR-GFP chimeric proteins.** The EpoR is schematically depicted, and extracellular, cytoplasmic, and TM domains are indicated. The *dark line* in the extracellular domain indicates the WS*X*WS motif. The *striped boxes* in the membrane-proximal portion of the cytoplasmic domain symbolize box 1 and box 2, where box 1 contains the continuous block of amino acids required for JAK2 binding. The *horizontal lines* in the membrane-distal portion indicate the eight tyrosine residues. The *dark circle* represents GFP that was inserted at the indicated positions in the EpoR. The *numbers* mark amino acid positions in the EpoR, and additionally introduced amino acids are indicated in single letter code.

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 stable 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. 2*A*). To evaluate whether GFP insertion affected surface transport of the chimeric receptors, we measured $^{125}$I-Epo binding to BaF3 cells stable expressing the EpoR derivatives. As shown in Fig. 2*B*, 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

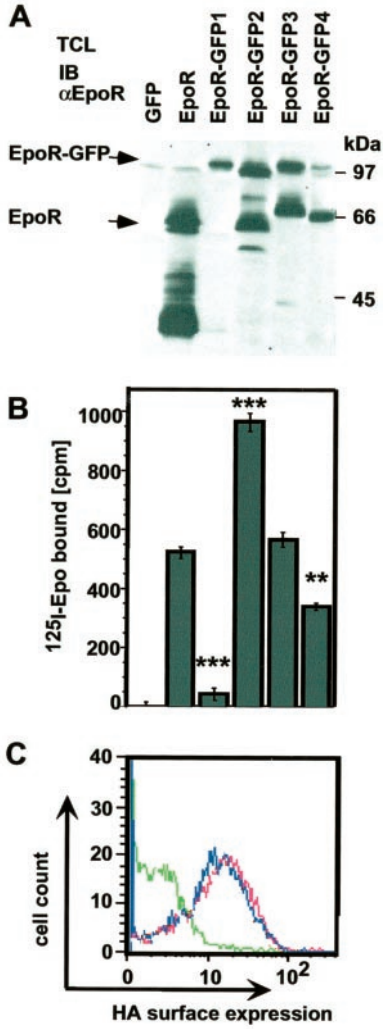


FIG. 2. **Cell surface expression of EpoR-GFP chimeric receptors.** *A*, total cell lysates were prepared from $2 \times 10^6$ BaF3 cells stable expressing GFP, the EpoR, or EpoR-GFP chimera and analyzed by immunoblotting with anti-EpoR antiserum. The *arrows* indicate the position of full-length EpoR and EpoR-GFP, whereas smaller bands represent cleavage products. *B*, saturation binding of $^{125}$I-Epo to BaF3 cell pools stable expressing GFP, the EpoR, or EpoR-GFP chimera. Surface expression was determined by the extent of $^{125}$I-Epo binding and plotted as specifically bound radioactivity (means ± S.D., $n = 3$). Significance was calculated with a two-sided paired Student's $t$ test compared with the values of wild type receptor (**, $p < 0.01$; ***, $p < 0.001$). The experiment was repeated three times with comparable results. *C*, cell surface expression of HA-tagged EpoR and EpoR-GFP1 and EpoR-GFP2. BaF3 cells stable expressing HA-EpoR (*blue line*), HA-EpoR-GFP1 (*green line*), and HA-EpoR-GFP2 (*red line*) were incubated with rat anti-HA antiserum followed by Cy5-labeled anti-rat IgG and analyzed by flow cytometry.

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. 2*C*). 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

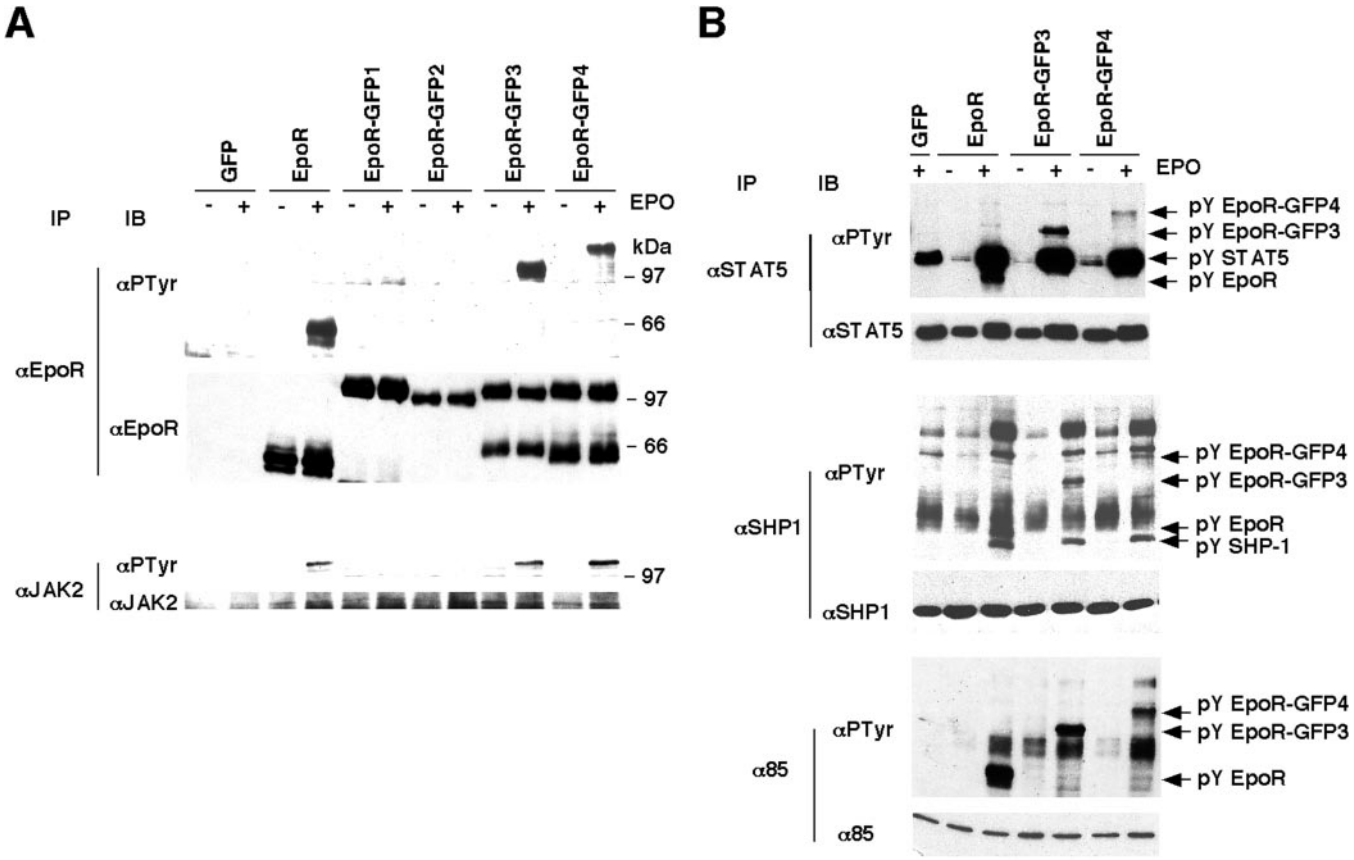


FIG. 3. **Activation of signal transduction by chimeric receptors harboring GFP inserted in the middle or fused to the C terminus of the cytoplasmic domain.** Starved BaF3 cells expressing GFP, the EpoR, or EpoR-GFP chimera were left unstimulated (−) or were stimulated with Epo (+), lysed, and subjected to immunoprecipitation (*IP*) with anti-EpoR and anti-JAK2 antiserum (*A*) or anti-STAT5, anti-SHP1, and anti-p85 antiserum (*B*). Immunoblotting analysis (*IB*) was performed using an anti-phosphotyrosine monoclonal antibody followed by detection with chemoluminescence. *B*, the positions of tyrosine-phosphorylated (*pY*) EpoR, EpoR-GFP3, EpoR-GFP4, STAT5, SHP-1, and p85 are indicated by *arrows*. Equal protein loading was ensured by reprobing the immunoblots with antibodies recognizing the respective proteins.

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. 3*A*). 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, SHP-1, and p85 (Fig. 3*B*). 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 chimera 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. 4*A* indicate that EpoR-GFP3 supported cell proliferation to a similar extent as wild type receptor, 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

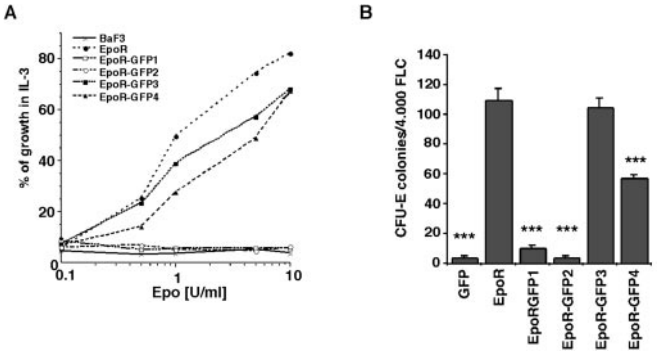


FIG. 4. **EpoR-GFP chimeric receptors capable of signal transduction support proliferation and differentiation.** *A*, proliferation of parental BaF3 cells or BaF3 cells expressing the EpoR or EpoR-GFP chimera in response to Epo. Cell numbers were determined using a Coulter counter. Growth is displayed 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.

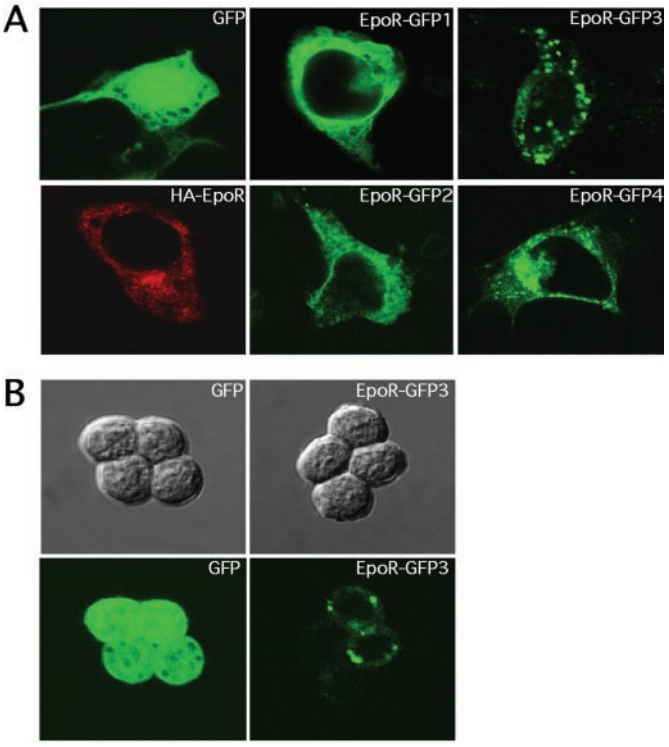


FIG. 5. **The EpoR localized to endosomal structures in living cells.** Confocal microscopy of GFP-EpoR and HA-EpoR in transfected 293T cells (*A*) and in transduced fetal liver cells (*B*). The cells were fixed with *para*-formaldehyde and permeabilized with 0.2% Triton X-100. HA-EpoR was detected with 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.

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 by confocal microscopy (Fig. 5*A*). The signaling-competent receptors EpoR-GFP3 and EpoR-GFP4 were detectable in intracellular structures resembling the ER, the Golgi, and punctated endosome-like structures. We performed overlay analysis of EpoR-GFP3 and a transiently expressed HA-tagged EpoR detected by anti-HA immunostaining. The HA-tagged EpoR is functionally indistinguishable from wild type EpoR (21) and showed similar subcellular localization (data not shown), thus confirming that the enrichment in punctated structures is not caused by GFP insertion. In erythroid progenitor cells, accumulation of EpoR-GFP3 in similar punctated structures was observed (Fig. 5*B*). Expression in other intracellular compartments was observed but was much dimmer compared with the bright endosome-like structures. The EpoR-GFP1 chimera that is unable to reach the cell surface predominantly resides within the ER network, and cells transfected with EpoR-GFP2, a receptor that is transported to the cell surface yet unable to trigger the activation of signaling, show an intermediate phenotype. The EpoR-GFP2 receptor predominantly remains in the ER, and only a minor portion is enriched in punctated structures.

Our analysis shows that the EpoR-GFP chimeric receptors facilitate the detection of EpoR and trafficking in living cells and therefore provide the possibility to visualize dynamic processes *in vivo*.

## 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 WS*X*WS motif in the EpoR extracellular domain, since deletion or alterations in the WS*X*WS motif resulted in receptors that were retained in the ER and unable to interact with the ligand (22). The WS*X*WS 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 WS*X*WS motif is located away from the interfaces that bind the respective ligand. The phenotype of the WS*X*WS 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. In this chimeric protein, GFP is localized in close proximity to $Tyr^{479}$, 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 $Tyr^{479}$ 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.

*Acknowledgments*—We thank Susanne Esser and Melanie Wickert for excellent technical assistance. The anti-p85 antiserum was generously provided by Lewis C. Cantley, and Stefan Constantinescu kindly provided the pMX-HA-EpoR-IRES-GFP. We thank Stephan Kuppig for help with the confocal microscope. We thank Dr. Hong Hu for providing EpoR knockout mice.

[2] R. Ketteler and U. Klingmüller, manuscript in preparation.

REFERENCES

1. Constantinescu, S. N., Ghaffari, S., and Lodish, H. F. (1999) *Trends Endocrinol. Metab.* **10,** 18–23
2. Klingmüller, U. (1997) *Eur. J. Biochem.* **249,** 637–647
3. Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jolliffe, L. K., and Wilson, I. A. (1999) *Science* **283,** 987–990
4. Kubatzky, K. F., Ruan, W., Gurezka, R., Cohen, J., Ketteler, R., Watowich, S. S., Neumann, D., Langosch, D., and Klingmuller, U. (2001) *Curr. Biol.* **11,** 110–115
5. Constantinescu, S. N., Huang, L. J., Nam, H., and Lodish, H. F. (2001) *Mol. Cell* **7,** 377–385
6. Huang, L. J., Constantinescu, S. N., and Lodish, H. F. (2001) *Mol. Cell* **8,** 1327–1338
7. Nicholson, S. E., and Hilton, D. J. (1998) *J. Leukocyte Biol.* **63,** 665–668
8. Klingmüller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995) *Cell* **80,** 729–738
9. Myers, M. P., Andersen, J. N., Cheng, A., Tremblay, M. L., Horvath, C. M., Parisien, J. P., Salmeen, A., Barford, D., and Tonks, N. K. (2001) *J. Biol. Chem.* **276,** 47771–47774
10. Irie-Sasaki, J., Sasaki, T., Matsumoto, W., Opavsky, A., Cheng, M., Welstead, G., Griffiths, E., Krawczyk, C., Richardson, C. D., Aitken, K., Iscove, N., Koretzky, G., Johnson, P., Liu, P., Rothstein, D. M., and Penninger, J. M. (2001) *Nature* **409,** 349–354
11. Kirito, K., Nakajima, K., Watanabe, T., Uchida, M., Tanaka, M., Ozawa, K., and Komatsu, N. (2002) *Blood* **99,** 102–110
12. Klingmüller, U., Wu, H., Hsiao, J. G., Toker, A., Duckworth, B. C., Cantley, L. C., and Lodish, H. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3016–3021
13. Damen, J. E., Cutler, R. L., Jiao, H., Yi, T., and Krystal, G. (1995) *J. Biol. Chem.* **270,** 23402–23408
14. Klingmüller, U., Bergelson, S., Hsiao, J. G., and Lodish, H. F. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8324–8328
15. Kirito, K., Uchida, M., Yamada, M., Miura, Y., and Komatsu, N. (1997) *J. Biol. Chem.* **272,** 16507–16513
16. Kirito, K., Uchida, M., Takatoku, M., Nakajima, K., Hirano, T., Miura, Y., and Komatsu, N. (1998) *Blood* **92,** 462–471
17. Gobert, S., Chretien, S., Gouilleux, F., Muller, O., Pallard, C., Dusanter-Fourt, I., Groner, B., Lacombe, C., Gisselbrecht, S., and Mayeux, P. (1996) *EMBO J.* **15,** 2434–2441
18. Ketteler, R., Glaser, S., Sandra, O., Martens, U. M., and Klingmüller, U. (2002) *Gene Ther.* **9,** 477–487
19. Wu, H., Liu, X., R., J., and Lodish, H. F. (1995) *Cell* **83,** 59–67
20. Levin, I., Cohen, J., Supino-Rosin, L., Yoshimura, A., Watowich, S. S., and Neumann, D. (1998) *FEBS Lett.* **427,** 164–170
21. Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H., Henis, Y. I., and Lodish, H. F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4379–4384
22. Yoshimura, A., Longmore, G., and Lodish, H. F. (1990) *Nature* **348,** 647–649
23. de Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* **255,** 306–312
24. Syed, R. S., Reid, S. W., Li, C., Cheetham, J. C., Aoki, K. H., Liu, B., Zhan, H., Osslund, T. D., Chirino, A. J., Zhang, J., Finer-Moore, J., Elliott, S., Sitney, K., Katz, B. A., Matthews, D. J., Wendoloski, J. J., Egrie, J., and Stroud, R. M. (1998) *Nature* **395,** 511–515
25. Remy, I., Wilson, I. A., and Michnick, S. W. (1999) *Science* **283,** 990–993
26. Neubauer, H., Cumano, A., Muller, M., Wu, H., Huffstadt, U., and Pfeffer, K. (1998) *Cell* **93,** 397–409
27. Parganas, E., Wang, D., Stravopodis, D., Topham, D. J., Marine, J. C., Teglund, S., Vanin, E. F., Bodner, S., Colamonici, O. R., van Deursen, J. M., Grosveld, G., and Ihle, J. N. (1998) *Cell* **93,** 385–395
28. Silverman, M. A., Kaech, S., Jareb, M., Burack, M. A., Vogt, L., Sonderegger, P., and Banker, G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7051–7057
29. Wouters, F. S., and Bastiaens, P. I. (1999) *Curr. Biol.* **9,** 1127–1130
30. Longmore, G. D., You, Y., Molden, J., Liu, K. D., Mikami, A., Lai, S. Y., Pharr, P., and Goldsmith, M. A. (1998) *Blood* **91,** 870–878