Structural Requirements of the Extracellular to Transmembrane Domain Junction for Erythropoietin Receptor Function*

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The erythropoietin receptor (EpoR) is crucial for erythrocyte formation. The x-ray crystal structures of the EpoR extracellular domain lack the juxtamembrane (JM) region and the junction to the transmembrane (TM) domain. Yet the JM-TM regions are important for transmitting the conformational change imposed on the receptor dimer by Epo binding. Cysteine-scanning mutagenesis of the JM-TM regions identified three novel constitutively active mutants, demonstrating close disulfide-bonded juxtapositioning of these residues in the JM (L223C) and N-terminal TM domain (L226C, I227C). Chemical cross-linking defined the interface of the active helical TM dimer and revealed that the JM-TM segment encompassing Leu226-Leu230 is non-helical. Molecular dynamics and NMR studies indicated that the TM-JM junction forms an N-terminal helix cap. This structure is important for EpoR function because replacement of this motif by consecutive leucines rendered the receptor constitutively active.

The erythropoietin receptor (EpoR) belongs to the cytokine receptor family (1), and along with its ligand erythropoietin (Epo), is the primary regulator of mammalian erythropoiesis (2, 3). Activation of the EpoR by Epo results in the activation of cytosolic Janus kinase 2 (JAK2) tyrosine kinase (4). Activated JAK2 phosphorylates tyrosine residues on itself, in various signaling molecules and in the EpoR cytoplasmic domain. These EpoR phosphotyrosines become docking sites for several signaling molecules and can activate the mitogen-activated protein kinase pathway. Phosphorylated EpoR recruits and activates STAT (signal transducers and activators of transcription) proteins, which are involved in the regulation of gene expression (5).

The EpoR has a large extracellular ligand binding domain, a single transmembrane (TM) helix, and a large cytoplasmic domain. The crystal structure of the extracellular ligand binding domain of the EpoR has been solved in complex with Epo (6), an agonist peptide (EMP1) (7), an antagonist peptide (EMP33) (8), and alone (9). These structures were dimeric and differed by the relative orientations of the extracellular domains. Receptor dimerization alone is not sufficient for activation as studies with dimerizing antagonists of Epo show that inhibitory dimeric conformations exist (8). In addition, we have recently shown that the relative dimeric orientation of the TM and cytoplasmic domains is also important for activation (10, 11). The unliganded EpoR is assumed to adopt a different conformation from the ligand-activated receptor, but both orientations are thought to be stabilized by interactions between the EpoR TM domains (12, 13).

The TM region of the EpoR stretches from roughly Leu226 to Leu237. These 22 mostly hydrophobic residues are sufficient to span the cell membrane. The TM region must adopt helical secondary structure in order to satisfy backbone hydrogen bonds within the hydrophobic membrane interior. The juxtamembrane sequence on the cytosolic side of the EpoR appears to be α-helical and contains within its first eleven amino acids a hydrophobic motif (Leu233, Ile235, Trp236), which is required for JAK2 activation (10). This motif is conformationally rigid, and its orientation is dictated by the register of the EpoR TM domain. Precise orientation of this motif is important for optimal signaling not only for the EpoR, but also for the gp130 subunit of the interleukin 6 receptor family (14).

In contrast, there are no data on the juxtamembrane region on the extracellular side of the membrane. It is not known where the transition between the extracellular domain and the TM α-helix occurs or whether this transition has a rigid structure. In this report, we define where that transition takes place by using several complementary approaches: single cysteine scanning mutagenesis and cross-linking, NMR spectroscopy, and molecular dynamics simulations. We show that three cysteine mutants in this region (L223C, L226C, I227C) result in constitutive activation as studies with dimerizing antagonists of Epo show that inhibitory dimeric conformations exist (8). In addition, we have recently shown that the relative dimeric orientation of the TM and cytoplasmic domains is also important for activation (10, 11). The unliganded EpoR is assumed to adopt a different conformation from the ligand-activated receptor, but both orientations are thought to be stabilized by interactions between the EpoR TM domains (12, 13).

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2 During the submission of this manuscript we became aware of an independent study by Xiaohui Lu and Harvey F. Lodish where the cysteine mutations at positions 223, 226, and 227 as well as other mutations in the transmembrane domain of the EpoR were isolated by random mutagenesis and selection for constitutive EpoR activation.

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1 The abbreviations used are: EpoR, erythropoietin receptor; STAT, signal transducer and activator of transcription; TM, transmembrane; HA, hemagglutinin; Tricine, N,4-(2-hydroxymethyl)-1,3-dimethylglycine; cc, coiled-coil; JAK2, Janus kinase 2; α-PDM, 1,2-diamino-5-imidophenylene; NEM, N-ethylmaleimide; DMPC, dimyristoyl-phosphatidylcholine; FACS, fluorescent-activated cell sorting; JM, juxtamembrane.

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EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—cDNAs were cloned into the pmX-IRESGFP bicistronic retroviral vector upstream of the IRES (15). In the wild-type HA-tagged murine EpoR cDNA Cys48 and Cys289 were mutated to Ser and Ala, respectively. By introducing a stop codon after amino acid 5, a free receptor molecule was created into which all single amino acid substitutions were cloned. cDNAs coding for the 28 single cysteine mutations in the EpoR juxtamembrane and transmembrane domains (A220C-L247C) as well as the Leu174 and Leu271 mutations were generated by overlap extension PCR with synthetic oligonucleotides. All constructs were verified by sequencing.

Cell Culture—Six-well plates of 283-derived human Bosc cells were transfected (Protueblo) with cDNA encoding for the empty plasmid, wild-type EpoR, or mutant receptors in the bicistronic vector pmX-IRESGFP as described (15). Cells were harvested 48 h after transfection for cross-linking experiments. To obtain BaF3 cell lines stably expressing the mutant receptors, retroviral supernatants were generated by co-transfection of the Bosc packaging cell line with receptor DNA and the eukaryotic vector pCL eco (17). 5 × 10⁵ BaF3 cells were centrifuged for 120 min at 37 °C and 900 × g in the presence of 16 µg of Polybrene and 750 µl of virus supernatant. After 48 h the transfection efficiency was evaluated by analysis of GFP expression.

Surface Expression of HA-EpoR—Surface expression of N-terminally HA-tagged EpoR was measured in BaF3 cells by flow cytometry using a monoclonal anti-HA antibody (Covance, 10 µg/ml) and R-phycocerythrin (PE)-conjugated donkey F(ab')2 anti-mouse IgG secondary antibody (Caltag, as described (18)).

Cross-linking Studies on Live Cells—Cells were washed with phosphate-buffered saline and resuspended in 1 ml of phosphate-buffered saline containing 1 mM MgCl₂ and 0.1 mM CaCl₂. If cysteines of interest were in the TM domain, extracellular cysteines were blocked by incubation with 100 µM N-ethylmaleimide (NEM) for 30 min at room temperature. NEM was removed by spinning the cells and aspiration of the supernatant. Cross-linking was then performed for 10 min at room temperature with a final concentration of the cross-linker 1,1-dimaleimido-polyethylene (ο-PDM) dissolved in Me₂SO of 100 µM. Cells were lysed with 1 x Nonidet P-40 lysis buffer in the presence of 2% β-mercaptoethanol to quench excess cross-linker. Lysate samples were separated on 10% Tricine SDS-PAGE gels, transferred to nitrocellulose, and detected by using a monoclonal actin antibody (Covance, 1:10,000 and detection by enhanced chemiluminescence (Amersham Biosciences). Actin was detected by using a monoclonal antibody (Sigma) and incubation with a mouse-herpesvirus peroxidase antibody (Amersham Biosciences). 101,000 and detection by enhanced chemiluminescence (Amersham Biosciences). Actin was detected by using a monoclonal antibody (Sigma) and incubation with a mouse-herpesvirus peroxidase antibody. For non-reducing gels, cells were lysed as described. Non-reducing Laemmli buffer was added to the lysates, and the proteins were separated by SDS-PAGE without prior boiling.

Assay for Growth Factor-independent Proliferation—Parental BaF3 cells and BaF3 cells expressing the wild-type EpoR, or the various constructs growing in IL-3 were washed extensively in RPMI medium, then placed in a 24-well plate with RPMI medium containing 10% fetal bovine serum without cytokines. Cell numbers were counted using a Coulter Counter after 1–10 days.

Dual Luciferase Assays—Functional STAT5 activation was assessed by measuring luciferase production of Bosc cells transfected with the plHRE-Luc (lactogenic hormone responsive element), which contains six copies of the prolactin STAT5 binding site in the rat β-casein promoter (19). As an internal control the pRL-TK vector (Promega, Madison, WI) was used. Bosc cells were seeded in 96-well plates at 5 × 10² cells/well and transfected with 50 ng of EpoR cDNA and 10 ng of the reporter DNA using the Lipofectamine transfectant. After 12 h, cells were stimulated with 100 units/ml Epo. Four hours after transfection, the cells were lysed, and luciferase assays were performed using the dual luciferase reporter assay kit (Promega).

Peptide Synthesis and Purification—Two peptides corresponding to the Epo receptor transmembrane domain (Asp-Leu-Asp-Pro-Val-Ile-Leu-Thr-Leu-Ser-Leu-Ile-Leu-Val-Leu-Leu-Leu-Thr-Val-Asp-Ala-Arg) were synthesized on an ABI 430A solid-phase peptide synthesizer (Applied Biosystems, Foster City, CA) using t-BOC chemistry. [1-13C]Lecine (Cambridge Isotope Laboratories, Andover, MA) was incorporated at either Leu² or Leu⁷ of the TM peptide sequence. Leu¹ and Leu² correspond to Leu229 and Leu236 of the murine EpoR sequence, respectively. The N and C termini were protected. The crude peptide (5–15 mg) was purified by reverse-phase high-performance liquid chromatography on a C4 column using gradient elution containing 0.1% trifluoroacetic acid. The three-gradient elution starts with a largely aqueous solution of 70% distilled water, 12% acetonitrile, and 18% 2-propanol alcohol, and is changed to a more hydrophobic composition of 40% acetonitrile and 55% 2-propanol alcohol that elutes the peptides. The elution was monitored by the optical absorbance at 220 nm. The purity was confirmed by mass spectrometry.

Epo receptor transmembrane peptides were reconstituted into DMPC (dimyristoylphosphatidylcholine) bilayers at a 50:1 lipid-to-peptide ratio. Purified EpoR peptides were reconstituted by detergent dialysis by first dissolving DMPC, lyophilized peptide, and detergent (octyl-β-glucoside) in trifluoroethanol. This mixture was incubated at 37 °C for over 1 h, then trifluoroethanol was removed by evaporation using a stream of argon gas and then placing the sample under vacuum. The dry mixture was rehydrated with phosphate buffer (10 mM phosphate and 50 mM NaCl, pH 7), so that the final concentration of octyl-β-glucoside was 5% (w/v). The rehydrated sample was stirred slowly for at least 6 h, and octyl-β-glucoside was removed by dialysis using SpectroPor dialysis tubing (3500 MW cutoff) for 24 h against phosphate buffer at 30 °C. The membrane-reconstituted peptides adopted helical transmembrane orientations as assessed by polarized IR spectroscopy. The reconstituted membranes were then pelleted to form multimellar dispersions and loaded into NMR rotors. Excess water was removed by spinning the sample in a tabletop rotor spinning unit.

Magic Angle Spinning NMR Spectroscopy—Solid-state MAS NMR measurements were performed on an Avance 500 MHz NMR spectrometer using a 4-mm double resonance probe (Bruker Instruments, Billerica, MA). [1-13C]spectra were acquired with ramped amplitude cross-polarization (20) and T1PPM proton decoupling (21). The 1H pulse length was 3.6 µs. The total contact time for cross polarization was 2 ms, and the recycle delay was 2 s. The proton decoupling field strength was 86 kHz. The 13C frequencies were referenced to the carbonyl resonance of solid glycerol at 184.90 ppm from DMSO. The MAS rotation frequency is 5.0 kHz ± 3 Hz and the temperature was maintained at ~30 °C. The spectra represent the average of ~60,000 scans and were processed without line broadening.

RESULTS

Cloning and Expression of Receptors with Single Cysteine Mutations—Cysteine-scanning analysis is a valuable and established method for predicting the topology of membrane proteins and to obtain information on parameters such as distance and conformation of introduced cysteine residues (26–28). This approach has been shown to be especially useful in the case of TM segments, where structural data are not easily obtained.

Introduction of cysteine residues at sequential positions allows characterization of interfaces, either by detection of spontaneously formed disulfide bonds or by chemical cross-linking. Formation of intermolecular disulfide bonds may lead to constitutively active receptors. An example is the EpoR R129C mutant, where replacement of an extracellular arginine with a cysteine leads to disulfide bond formation and constitutive activation (29, 30).

We constructed an HA-tagged EpoR in the retroviral pmX-IRESGFP vector, fully cotransfected with the only four conserved cysteine residues of the EpoR extracellular domain, that are indispensable for protein folding and ligand binding (Fig. 1A). These cysteines are thought to form two intramolecular disulfide bonds between residues Cys²⁸ and Cys³⁰, and Cys⁶⁰ and Cys² respectively (30). A fifth cysteine residue in the extracellular domain, Cys₁⁶⁰, was replaced by the related amino acid serine. In the intracellular sequence, Cys²⁸ was mutated to alanine and the cytoplasmic tail was truncated by introduction of a stop
codon after Pro\textsuperscript{355}. As a result, this truncated receptor, trEpoR\textsubscript{4Cys}, does not contain cysteines that should be available for sulphydryl cross-linking, but includes Tyr\textsuperscript{343}, that is known to be one of the two STAT5 binding sites (31, 32). Into this thiol-free background, as well as into the wild-type EpoR, single amino acid substitutions to cysteine were introduced consecutively from Ala\textsuperscript{220} to Leu\textsuperscript{247} (Fig. 1, \textit{a} and \textit{b}).

Transiently transfected human 293-derived Bosc cells were tested for expression of the receptor mutants. Immunoblotting of cell lysates with an EpoR antibody revealed that all receptors were expressed equally well with the expected size of 41 kDa for truncated receptors (Fig. 3 \textit{b}) and 68 kDa for full-length receptors, respectively, and were able to respond to Epo by activation of STAT5, as verified by luciferase assays (data not shown).

**Cysteine Scanning of the Juxtamembrane Sequence and Identification of Constitutively Active Receptors**—The mutant receptors were then examined for the induction of biologic effects in murine BaF\textsubscript{3} cells. This proB cell line requires IL3 for survival and proliferation but can be rendered Epo-dependent upon transduction of the EpoR (33). Retroviral supernatants were prepared as described previously (3, 16) for infection of parental BaF\textsubscript{3} cells. Stable cell lines expressing the mutant receptors were established and tested for infection efficiency, i.e. GFP expression, and expression of the receptors at the cell surface by HA staining and subsequent FACS analysis. For all receptors the ratio between receptors expressed at the cell surface and the infection efficiency, as determined by GFP expression, was at levels comparable to the wild-type receptor (Fig. 2 \textit{c}).

BaF\textsubscript{3} cells expressing the different EpoR cysteine point mutants were tested for their ability to grow in medium containing only 10\% fetal serum. As a control, the mutant EpoR R129C was used, which is known to allow factor-independent cell growth (29, 30, 34). Of the 28 cysteine mutants introduced into the wild-type receptor, only L223C, L226C, and I227C were able to grow in the absence of IL3 or Epo. Fig. 2\textit{a} shows that the kinetics of ligand-independent growth after selection of cells for 1 week without growth factor is the same for R129C-expressing cells and cells harboring a mutation at L223C, L226C, or I227C. The selection for constitutive activation can be detected as an enrichment for the percentage of cells that express GFP (Fig. 2 \textit{b}).

The constitutive activity of EpoR L223C, L226C, and L227C is likely caused by formation of intermolecular disulfide bonds that fix the EpoR in a conformation that is productive for signaling. To test this hypothesis we assayed their migration in non-reducing (Fig. 3 \textit{a}) and reducing (Fig. 3 \textit{b}) Tricine gels and by immunoblotting. As shown in Fig. 3\textit{a}, strong and equivalent disulfide bonds were formed by A220C to D224C and L226C mutants. P225C, I227C, and L228C formed disulfide bonds with reduced efficiency. None of the other mutants from T229C to L247C formed dimers. Thus, there is no correlation between disulfide bond formation and activity, as the adopted conformation of inactive dimers may not be productive for signaling. This was also observed in studies involving the Neu (Erb-2) receptor, where only a subset of the receptors showing constitutive dimerization because of an engineered cysteine mutation had transforming activity (35).

In non-reducing Tricine gels, besides the 100-kDa complex which was detected for trL223C, trL226C, and trI227C, an additional 90-kDa double band was observed for all receptors including the negative control trEpoR\textsubscript{4Cys}. This double band corresponds to receptor dimers formed via the four extracellular cysteines in the unglycosylated and glycosylated forms of the receptor (see below and Fig. 4\textit{c}). Indeed, under non-reducing conditions, these conserved cysteines are able to participate in the formation of higher oligomeric complexes of unknown stoichiometry (36). The 100-kDa complex detected for the trL223C, trL226C and trI227C mutants is a specific product of

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**FIG. 1.** Schematic presentation of receptors for single cysteine-scanning mutagenesis. \textit{a}, pMX-IRES-GFP HA trEpoR\textsubscript{4Cys} represents the backbone receptor for the introduction of single cysteine residues in the TMD and subsequent cross-linking studies. The receptor contains only the four conserved extracellular cysteines (Cys\textsuperscript{28}, Cys\textsuperscript{38}, and Cys\textsuperscript{66}, Cys\textsuperscript{88}), Cys\textsuperscript{180} and Cys\textsuperscript{289} are mutated to serine and alanine, respectively. The receptor is truncated by introduction of a stop codon after Pro\textsuperscript{355} and contains an N-terminal HA tag. \textit{b}, pMX-IRES-GFP HA EpoR represents the wild-type, full-length vector containing an N-terminal HA tag. This receptor was used in proliferation experiments.
receptors whose TM helices are in close proximity (app. 3 Å). The differences in apparent molecular weight between the EpoR mutants and the wild-type receptor are presumably caused by differences in the shape of the cross-linked dimer and depend on the location of disulfide bond formation. In this regard, the trR129C mutant can be detected under non-reducing conditions as an 95 kDa dimer (data not shown). Addition of -mercaptoethanol to the samples reduced all oligomeric complexes to the monomeric forms that appear as one single band under the experimental conditions used (Fig. 3b).

Cross-linking of Receptors in Cells with a 9-Å Spacer—Truncated receptors containing single cysteine mutations in the background of trEpoR4Cys were transiently transfected into Bosc cells and subjected to cross-linking with the sulfhydryl-specific, membrane-permeable, homobifunctional cross-linker (1, 2)-dimaleimidylphenylene (9-PDM) on living cells (Fig. 4, a and b). As the two maleimide groups of 9-PDM are coupled to an aromatic ring, the distance spanned by the cross-linker does not vary much in solution, and allows the linkage of sulfhydryl groups separated by a distance of 9.4 ± 0.5 Å (37). 9-PDM has the shortest spacer of the chemical cross-linkers tested and resulted in a more distinct pattern of cross-linking than cross-linkers with a longer spacer arm (data not shown).

We assayed the sequence from Ala220 to Leu247, which includes both the extracellular juxtamembrane region and the TM domain of the EpoR. Most secondary structure prediction algorithms predict that Pro225 is the last amino acid in the juxtamembrane region so that the membrane-spanning -helix stretches from Leu226 to Leu247. Only the algorithm developed by Rost et al. (38) predicts that the juxtamembrane region has an extended structure through Pro225 to Thr229 and that the TM helix begins at Leu230 (10).

To inhibit any reaction of the extracellular cysteines with the cross-linker, receptors harboring cysteine mutations in the transmembrane domain (L226C-L247C, Fig. 4a) were first incubated with the water-soluble reagent N-ethylmaleimide (NEM), that blocks free cysteines. All cells were then cross-linked with 9-PDM, lysed with Nonidet P-40 buffer and an aliquot of the lysate was used for the analysis on reducing 10% Tricine gels (Fig. 4, a and b).

Immunoblotting with an EpoR antibody shows that mutants L226C-L230C cross-link without any periodicity (Fig. 4a), while Fig. 4b shows that indeed the entire region L220C to L230C cross-links without interruption, which is incompatible with the prediction that the TM domain extends through Pro225. Besides their monomer and dimer bands, receptors that were not treated with NEM reveal an additional 90 kDa (Fig. 4b). Again, this double band represents cross-linking via the four conserved cysteines in the extracellular part of the receptor (see above non-reducing gels,
Fig. 3. Expression of receptor mutants. a, detection of EpoR dimers under non-reducing conditions. Bosc cells were transfected with DNA coding for the empty vector, trEpoR4Cys or the various single cysteine mutant receptors (trA220C-trL247C), lysed, and run on a 10% Tricine gel before immunoblotting with an EpoR antibody. Arrows indicate the size of the monomer and the dimeric proteins. Formation of unspecific intermolecular disulfide bonds to the four extracellular cysteines are marked by a bracket and can be distinguished from transmembrane domain disulfide bond formation by a difference in size (90 versus 100 kDa, respectively). b, for control of expression levels by Western blotting, lysates were prepared as described and run on a 10% Tricine gel under reducing conditions before immunoblotting with an EpoR antibody to detect EpoR monomers.

From our cross-linking results we conclude that the first five amino acids of the predicted TM domain (Leu226-Leu230) are not α-helical and rigid. In addition to residues belonging to the non-helical motif (Leu224-Leu226), only residues L232C, L241C, L244C, and L246C could be cross-linked with o-PMD (Fig. 4a). This result was obtained both when cells were lysed in buffer containing detergent (1% Nonidet P-40), and also for lysates that were obtained by directly boiling cells in reducing Laemmli buffer (not shown). As a result, cross-linking must have occurred in native membranes and was not an artifact of detergent solubilization.

In summary, we show that the same EpoR mutants (L223C, L226C, and I227C) that exhibit constitutive activation also cross-link with o-PDM. In addition, residues 220–222, 224, 225, and 228C-230C cross-link with o-PDM, but do not exhibit constitutive activation. It is interesting that none of the other cross-linked cysteine mutants at the C-terminal end (e.g. L241C, L244C, A245C) of the TM domain are able to grow in the absence of ligand, although cells expressing the EpoR L244C mutant are significantly protected from apoptosis (not shown) upon withdrawal of cytokines. Dimerization at the level of the TM domain may for example not allow sufficient flexibility of the cytosolic domains, which are required for assembly of signaling complexes. This was reported for the epidermal growth factor receptor, where activation involves a rotation of the TM domains due to the flexibility of the extracellular juxtamembrane region (39). For the EpoR, previous reports (11), as well as our cross-linking studies, pinpointed to Leu241 and Leu244 as a critical region for receptor dimerization. Replacement of Leu241 by charged residues (40) or introduction of a glycine-proline mutation (13) at Leu240 and Leu244 negatively affects receptor function.

Helix Capping at Thr229-Leu230—Our disulfide cross-linking results indicate that the five first residues of the predicted TM domain (Leu226-Leu230) are not α-helical and rigid. In addition to residues belonging to the non-helical motif (Leu224-Leu226), only residues L232C, L241C, L244C, and to a lesser extent A245C, and very weakly L246C could be cross-linked with o-PMD (Fig. 4a). This result was obtained both when cells were lysed in buffer containing detergent (1% Nonidet P-40), and also for lysates that were obtained by directly boiling cells in reducing Laemmli buffer (not shown). As a result, cross-linking must have occurred in native membranes and was not an artifact of detergent solubilization.

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Helix Capping at Thr229-Leu230—Our disulfide cross-linking results indicate that the five first residues of the predicted TM domain (Leu226-Leu230) are not α-helical and rigid. In addition to residues belonging to the non-helical motif (Leu224-Leu226), only residues L232C, L241C, L244C, and to a lesser extent A245C, and very weakly L246C could be cross-linked with o-PMD (Fig. 4a). This result was obtained both when cells were lysed in buffer containing detergent (1% Nonidet P-40), and also for lysates that were obtained by directly boiling cells in reducing Laemmli buffer (not shown). As a result, cross-linking must have occurred in native membranes and was not an artifact of detergent solubilization.

In summary, we show that the same EpoR mutants (L223C, L226C, and I227C) that exhibit constitutive activation also cross-link with o-PDM. In addition, residues 220–222, 224, 225, and 228C-230C cross-link with o-PDM, but do not exhibit constitutive activation. It is interesting that none of the other cross-linked cysteine mutants at the C-terminal end (e.g. L241C, L244C, A245C) of the TM domain are able to grow in the absence of ligand, although cells expressing the EpoR L244C mutant are significantly protected from apoptosis (not shown) upon withdrawal of cytokines. Dimerization at the level of the TM domain may for example not allow sufficient flexibility of the cytosolic domains, which are required for assembly of signaling complexes. This was reported for the epidermal growth factor receptor, where activation involves a rotation of the TM domains due to the flexibility of the extracellular juxtamembrane region (39). For the EpoR, previous reports (11), as well as our cross-linking studies, pinpointed to Leu241 and Leu244 as a critical region for receptor dimerization. Replacement of Leu241 by charged residues (40) or introduction of a glycine-proline mutation (13) at Leu240 and Leu244 negatively affects receptor function.
pletely extended conformations folded to partially helical structures with Leu230 as a helical cap. Fig. 5a illustrates the representative structure obtained from cluster analysis of the simulation at 300 K. As Aurora and Rose (41) point out, helix capping typically involves both hydrophobic and hydrogen bonding interactions. This is seen in the hydrophobic contact that Leu228 has with Leu232 and Ile233, and hydrogen bonding of the Thr229 side chain back to the amide NH of Leu232. This hydrogen bond is present in 60% of the structures sampled during the simulation (Fig. 5a). In Fig. 5b, the average fractional helical content for each residue in the sequence is shown for simulation at 300 K. The N-terminal region shows individual residues that sample helical conformations, but no stretches of more than two helical residues are evident. In contrast, all residues after Leu230 have 50–80% average helical content, with fraying of the helix evident at the C-terminal residues. This pattern is similar at 325 K (data not shown), with the transition between unstructured and helical regions again occurring at Leu230.

Additional support for the N-cap structure comes from solid-state magic angle spinning (MAS) NMR measurements of peptides corresponding to the TM and juxtamembrane regions of the EpoR. Specifically, we compared the NMR spectra of peptides 13C-labeled at the backbone carbonyl of Leu228 or Leu230. The 13C chemical shift of the backbone carbonyl is sensitive to hydrogen bonding and the conformation around the carbonyl-Cα bond (42, 43). Amino acids in helical secondary structure exhibit 13C chemical shifts in the range of 175–176 ppm, while extended β-strand and β-sheet secondary structure have chemical shifts below ~172 ppm. Leu230 is the last amino acid in helical secondary structure in the N-cap structure shown in Fig. 5a.

The MAS NMR spectrum of the EpoR TM peptides 13C-labeled at Leu230 is shown in Fig. 5d (top). Only the carbonyl region of the spectrum is shown, along with a deconvolution into Gaussian components. The experimental spectrum (black) exhibits two resonances. The resonance at 172 ppm corresponds predominantly to the natural abundance 13C signal from the lipid carbonyls. The natural abundance 13C background was independently fit in MAS spectra of lipid and unlabeled peptide to two Gaussian components (blue). The resonance at 175.3 ppm corresponds to the [1-13C]carbonyl carbon of Leu230. The deconvoluted [1-13C]carbonyl resonance is shown in red. The chemical shift is characteristic of α-helical secondary structure. The NMR spectrum of L228 (Fig. 5d, bottom) on the other hand is dramatically different, as no sharp resonance corresponding to an α-helix is observed. Using the same 13C natural abundance background for the fit of the line shape, the [1-13C]carbonyl resonance (red) is instead broadened and shifted to ~172 ppm, consistent with the loss of defined helical secondary structure.
Replacement of the Helix Cap by an Oligoleucine Segment—To assess the importance of the helix cap motif for the activation and signaling of the EpoR, we replaced the first six or nine amino acids of the TM domain by leucines (Leu6, Leu9). This was accomplished by mutating to leucine residues Ile227, Thr229, Ser231, and Ile233 as the other four residues of the cap structure are originally leucine (see Fig. 6a). Structural and functional assays had shown that oligoleucine motifs are helical and capable of efficient dimerization within an artificial transmembrane because of the packing of $\alpha$-helices in a leucine zipper type of interaction (44). We hypothesized that replacement of the helix cap by leucines would elongate the helix up to Leu226 and result in a different mode of activation because of a different pitch of the downstream helix and possibly enforced self-assembly. Thus we infected BaF3 cells with viruses for either the wild-type receptor, Leu6 or Leu9 and the R129C mutant as a control. Cells were selected for their ability to grow in the absence of ligand or in a dose-dependent response to Epo. No difference could be seen for Leu6 or Leu9 in the response to increasing amounts of Epo after 48 h of growth, when compared with the wild-type receptor (data not shown). Nevertheless, during selection of cells in 10% fetal serum for 10 days, replacement of the first six residues (Leu226-Leu230) resulted in a significant number of cells that survived longer than cells expressing wild-type EpoR, but proliferated poorly and eventually died, so that no difference in growth could be detected by counting. On the other hand, cells expressing the receptor, where the proximal region of the predicted TM domain was replaced by an $\alpha$-helical structure composed of nine leucines (Leu226-Leu234), i.e. the complete helix cap motif, the receptor showed ligand-independent activation (Fig. 6b). The sole difference between Leu6 and Leu9 is residue Ile233, which is an isoleucine in Leu6 and leucine in Leu9. Molecular dynamics simulations indicate that Ile233 forms hydrophobic interactions with the cap residue Leu228. These results suggest that the $\beta$-branched isoleucine residue plays an important role in stabilizing the cap structure and the unliganded inactive dimeric conformation of the receptor.

In summary these data give convincing evidence that the first nine amino acids of the EpoR TM domain are involved in the formation of a helix cap, and that this motif allows the EpoR to be activated in a ligand-dependent manner, whereas the TM helices presumably stabilize the dimeric interface of the active receptor. Deletion of the cap motif by extension of the TM $\alpha$-helix leads to constitutive activation and uncontrolled cell proliferation.

Amino Acid Insertions into the Juxtamembrane Region of the EpoR—To test whether the structure near Pro225 is flexible we inserted one glycine residue between Asp224 and Pro225, either in the context of the wild-type receptor (EpoR-GP) or the constitutively active receptor mutant R129C (R129C-GP). The combination of these two residues should only be tolerated in the context of a flexible structure. Introduction of a GP mutation in the TM $\alpha$-helix disrupts its correct structure and de-
creases tyrosine phosphorylation of JAK2 and the receptor. In the background of the R129C mutant, this mutation abrogates ligand-independent signaling (13).

To investigate the receptors’ capability to activate the JAK-STAT signaling pathway, we transfected these receptors into Bosc cells. Cells were stimulated with 100 units/ml Epo, and the transcriptional activity was assessed by quantification of luciferase activity. As shown in Fig. 7 there was no major difference induced in the wild-type EpoR or the R129C mutant by this insertion. EpoR and EpoR-GP show comparable STAT5 reporter activity. Both R129C and R129C-GP show strong activation of STAT5 in the absence of ligand that can be further increased by addition of Epo. We infected BaF3 cells with virus coding for wild-type receptor EpoR, the constitutive receptor R129C, and the GP mutants EpoR-GP and R129C-GP and investigated their proliferation in the absence of Epo or with various Epo concentrations. No major difference was detectable in the ability to proliferate in the presence of Epo, and cells expressing R129C-GP could still grow in the absence of Epo (not shown). This result supports the hypothesis that the extracellular juxtamembrane region is not α-helical, but must be part of a rather flexible structure.

Replacement of the EpoR Extracellular Domain by the Coiled-coil (cc) of Put3—Recently we have investigated the ability of distinct dimeric conformations of the EpoR to signal. The extracellular domain of the EpoR was replaced by the Put3 dimeric cc domain, which has an amphipathic structure stabilized by hydrogen bonding and hydrophobic interactions (45, 46). It imposes its coiled-coil geometry on a downstream TM register of the coiled-coil is known, fusion with downstream helices with different junction points will impose all seven relative orientations to the EpoR TM. Two of these fusion proteins (cc-EpoR-III and cc-EpoR-VI) define an active dimeric interface and mimic signaling by Epo-activated EpoR (11). Residue L244 is predicted to be in the interface of both active constructs, cc-EpoR-III and cc-EpoR-VI, (an α or d position of the coiled-coil α-g heptad repeat) and in cc-EpoR-III, Leu241 and Leu244 are predicted to be in the interface (11). Since both L241C and L244C appear as cross-linked bands in the present study (Fig. 4a), we suggest that this is representative of the activated EpoR dimeric interface.

Thus we used the cc-EpoR fusion protein system to ask whether cysteine-mediated cross-linking could be used to show that Leu244 is in the interface of only the active cc-EpoR-III and cc-EpoR-VI fusion proteins and not of any of the others, cc-EpoR-0, I, II, IV, and V. Second, we tested whether the N-terminal five amino acid residues Leu226-Leu230 maintain their non-helical structure found in wild-type EpoR, or adopt a helical structure because of the upstream coiled-coil. We constructed fusion proteins that resemble the seven described cc-EpoR constructs (cc-EpoR-0-VI), but have residue Leu244 of the TM domain mutated to cysteine (L244C) and which contain the truncated thiol-free cytosolic domain as in cysteine-dependent oligomerization studies (Fig. 1a). Because of its close juxtaposition in the dimeric interface the presence of the L244C mutation specifically induced cross-linker-independent disulfide bond formation and dimerization in the wild-type EpoR and presents therefore a very sensitive assay to test the distance of receptor subunits in the dimeric interface (data not shown).

The receptors were transiently transfected into Bosc cells, lysed, and analyzed on 16.5% Tricine gels. All fusion proteins were expressed with the expected size of 18 kDa. Consistent with our hypothesis that the two active fusion proteins cc-EpoR-III and cc-EpoR-VI should have L244C in the dimeric interface, we were able to detect formation of disulfide-bonded dimers in the absence of cross-linker for these two receptors (Fig. 8a). As non-reducing conditions lead to unspecific dimerization due to the coiled-coil domain, reducing conditions were used without prior boiling that allowed to detect the stronger interaction between the disulfide-bonded cysteines at residues 244C. The agreement between the results obtained with engineered fusion proteins and those obtained with cysteine mutants of the wild-type EpoR argue strongly that Leu244 is in the interface of the activated EpoR dimer and that the cc-EpoR fusion proteins indeed induce the predicted interface of the downstream helices.

To answer the second question, we mutated each of the five residues, Leu226, Ile227, Leu228, Thr229 and Leu230 to cysteine and compared their o-PDM-induced cross-linking in the context of the wild-type EpoR and in the context of the Put3-cc-EpoR fusion protein cc-EpoR-0, which contains all TM residues. All residues in this motif cross-link with o-PDM in the context of
the wild-type EpoR (Fig. 4a), but only one residue (L226C) cross-links in the context of the coiled-coil fusion protein cc-EpoR-0 (Fig. 8b). This is typical of an extended structure for the Leu226-Leu230 region in the wild-type EpoR, but the latter results are characteristic for an $\alpha$-helical structure of the same region in cc-EpoR-0 context. Because the last residue of Put3 is in a $b$ position, only one interface ($a$ or $d$) residue can be predicted to exist among the following five residues in the cc-EpoR-0 context. Indeed, Fig. 8b shows that only one residue, L226C, is able to cross-link. Based on prediction, the best candidate for cross-linking would have been residue 227. However, it is possible that introduction of a cysteine rotates the $\alpha$-helix when it is fused to the coiled-coil so that cross-linking occurs at the preceding position, or that the intrinsic tendency to form an extended structure for the sequence Leu$^{226}$-Leu$^{230}$ distorts the helix and brings Leu$^{226}$ in the interface. Nevertheless, the patterns of cross-linking are strikingly different in the wild-type EpoR and Put3 proteins for the same sequence. This is reminiscent of studies, which showed that identical sequences adopt different structures as a function of context (47). The rotation/distortion effect of Leu$^{226}$-Leu$^{230}$ sequence is relevant only for cc-EpoR-0, since the other cc-EpoR fusion proteins contain N-terminal truncations of the EpoR TM domain, such as the active fusion proteins cc-EpoR-III and cc-EpoR-VI, which lack the first three or six TM domain residues, respectively. In addition, this effect may be responsible for the unusual properties of cc-EpoR-0, which induces cell survival and mitogen-activated protein kinase activation but fails to induce cell proliferation andSTAT activation (11).

DISCUSSION

Our main observations are that introduction of cysteine residues in the EpoR juxtamembrane region (L223C), and transmembrane region (L226C, I227C) results in constitutive activation of the receptor and that the junction between the JM and TM domains is not helical, forms a helix cap instead and that its precise sequence is important for proper receptor function. The approach typically taken for high resolution structural studies of cytokine receptors and receptor tyrosine kinases is to independently determine the structures of their extracellular, transmembrane and intracellular domains. The juxtamembrane regions flanking the TM domain are generally not tar-

**FIG. 7.** Mutational analysis of the juxtamembrane region connecting extracellular and transmembrane domain. Quantification of STAT5 transcriptional activity by the luciferase assay. Bosc cells were transfected with receptor DNA, JAK2, STAT5, p.LHRE-Luc, and pRL-TK in order to allow normalization of luciferase activity. Cells were stimulated for 4 h with 100 units/ml Epo, lysed, and assayed for luciferase activity. Results were obtained from one representative experiment performed in triplicates ± S.D.

**FIG. 8.** Replacement of the extracellular domain by the Put3 coiled-coil. a, Bosc cells were transiently transfected with DNA coding for chimeric receptors where the tr244C receptor extracellular domain and hinge region was replaced by the Put3-EpoR deletion mutants cc-EpoR-0 to cc-EpoR-VI. Lysates were analyzed for their dimerizing capacity on 16.5% Tricine gels by subsequent immunoblotting with an HA antibody. b, Bosc cells were transiently transfected with DNA coding for chimeric receptors where the Put3-EpoR mutant cc-EpoR-0 was fused to the cytoplasmic domains of tr226C-tr230C. Lysates were analyzed on 16.5% Tricine gels for their dimerizing capacity by cross-linking with o-PDM and immunoblotting with an HA antibody.
geted, yet are of intense interest because they couple ligand binding within the extracellular domain to conformational changes in the transmembrane and cytoplasmic domains.

Here, we show that the sequence linking the extracellular domain of the EpoR to the TM domain is extended and that the first amino acid residues of the predicted TM domain form a helix cap. Moreover, we describe three EpoR cysteine point mutants in the extracellular juxtamembrane region, L223C, L226C, and I227C, which induce constitutive activity by formation of disulfide-linked dimers. We show that insertion of glycine before Pro229 is well tolerated and that replacement of the first nine residues of the EpoR TM domain with consecutive leucines leads to constitutive receptor activation. These observations are in agreement with proposed activation mechanisms based solely on close proximity of EpoR monomers, and would be consistent with the ability to form active EpoR dimers in a number of very different ways, i.e., with mimetic antibodies (48), the R129C mutation (29), agonist peptides (49) and co-expression of the gp55 envelope protein of the Spleen Focus Forming Virus (50–52).

However, there is increasing evidence that specific orientations of EpoR monomers in the dimers complex are required for activation. For example, peptide antagonists have been described that hold the receptor extracellular domains in an inactive orientation (8). Moreover, insertion of a GP motif in the TM domain is detrimental for receptor activation. These results indicate that the TM domain must have a specific orientation for signaling.

The extended JM structure and the ability to cross-link the sequence from positions 220 to 230 suggest that the JM region has some flexibility. Intermolecular contacts between JM residues may be responsible for stabilizing inactive or active conformations in dimers of the wild-type receptor. The extracellular juxtamembrane sequences may be accessible to small molecule drugs that would target this region and activate receptors via different sites than those utilized by ligands.

By replacing the extracellular domain of the EpoR with the coiled-coil dimerization domain of the yeast transcription factor 12, Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H., Henis, Y. I., and Lodish, H. F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4379–4384. the JM region may reflect a combination of inactive and active dimer conformations or an asymmetric dimer interface for the activated receptor (6). Ongoing experiments are attempting to distinguish between these possibilities.

In summary our data on the structure and function of the EpoR JM and TM domains support the concept that precise TM sequences are necessary for proper receptor activation and demonstrate that close apposition between residues bordering the TM domain is possible in the context of the cysteine containing mutants, leading to disulfide-bonded EpoR-activated dimers.

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