Active Conformation of the Erythropoietin Receptor

**RANDOM AND CYSTEINE-SCANNING MUTAGENESIS OF THE EXTRACELLULAR JUXTAMEMBRANE AND TRANSMEMBRANE DOMAINS**

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In the absence of erythropoietin (Epo) cell surface Epo receptors (EpoR) are dimeric; dimerization is mediated mainly by the transmembrane domain. Binding of Epo changes the orientation of the two receptor subunits. This conformational change is transmitted through the juxtamembrane and transmembrane domains, leading to activation of JAK2 kinase and induction of proliferation and survival signals. To define the active EpoR conformation(s) we screened libraries of EpoR with random mutations in the transmembrane domain and identified several point mutations that activate the EpoR in the absence of ligand, including changes of either of the first two transmembrane domain residues (Leu226 and Ile227) to cysteine. Following this discovery, we performed cysteine-scanning mutagenesis in the EpoR juxtamembrane and transmembrane domains. Many mutants formed disulfide-linked receptor dimers, but only EpoR dimers linked by cysteines at positions 223, 226, or 227 activated EpoR signal transduction pathways and supported proliferation of Ba/F3 cells in the absence of cytokines. These data suggest that activation of dimeric EpoR by Epo binding is achieved by reorienting the EpoR transmembrane and the connected cytosolic domains and that certain disulfide-bonded dimers represent the activated dimeric conformation of the EpoR, constitutively activating downstream signaling. Based on our data and the previously determined structure of Epo bound to a dimer of the EpoR extracellular domain, we present a model of the active and inactive conformations of the Epo receptor.

The cytokine erythropoietin (Epo) is the primary regulator of mammalian erythropoiesis. The erythropoietin receptor (EpoR), a member of the cytokine receptor family, is comprised of an extracellular ligand binding domain, a single transmembrane domain, and an intracellular domain (1). Epo binding triggers trans-phosphorylation and activation of the Janus family protein tyrosine kinase JAK2 that is appended to the intracellular domain of EpoR. Activated JAK2 then phosphorylates tyrosine residues in EpoR, creating docking sites for intracellular signaling proteins such as STAT5, phosphatidylinositol 3'-kinase, and SHP1. These events lead to the activation of several signal transduction pathways and specific gene expression, resulting in the survival, proliferation, and differentiation of erythroid progenitors (2). Without Epo or EpoR, definitive erythropoiesis cannot occur (3).

Previous studies on a constitutively activated EpoR mutant, R129C, revealed that the activated EpoR is a dimer (4). Recent evidence indicates that surface EpoR exists as a dimer in the absence of Epo, and that Epo binding likely activates the EpoR through defined conformational changes (5, 6). The EpoR extracellular segment consists of two fibronectin III domains, D1 and D2. The crystal structure of two EpoR extracellular ligand binding domains (Epo-binding protein, EBP) bound to one Epo molecule showed that the D1 domains of the two dimerized EpoRs are positioned at a 120° angle when viewed perpendicular to the membrane plane (7). However, the crystal structure of the EpoR extracellular domains bound to a less potent Epo mimic peptide (EMP) assumes a symmetric orientation with a 180° angle between the two D1 domains (8). This suggests that there is more than one conformation that can activate EpoR signaling, and that a 120° angle between the two receptors may be important for optimal signaling (7). We hypothesize that Epo activates EpoR by positioning the two EpoR molecules in a particular dimeric conformation, which brings the active site of one (poorly active) JAK2 kinase immediately adjacent to the critical tyrosine residue in the activation loop of the second JAK2. By phosphorylating this tyrosine, the second JAK2 becomes catalytically active, triggering all downstream signaling events.

EpoR dimerization in the absence of ligand is mediated mainly by its transmembrane domain (5) and the EpoR transmembrane domain is a membrane-spanning leucine zipper that strongly induces dimer formation (9). Abrogating its dimerization by introducing mutations in the transmembrane domain impaired EpoR signaling in mammalian cells (9, 10). The EpoR cytosolic juxtamembrane domain contains a highly conserved hydrophobic motif (Leu223, Ile227, and Trp230) that is required for JAK2 activation (11). Experiments in which 1, 2, 3, or 4 alanines were inserted between the transmembrane segment and this motif suggested that the transmembrane and cytosolic juxtamembrane domains of EpoR form a continuous and rigid α-helix (12). This rigid structure may be important for “locking” the unliganded dimeric EpoR in an inactive conformation and thus preventing adventitious, inappropriate receptor activation.

Clearly the EpoR transmembrane and juxtamembrane segments play key roles in properly orienting the EpoRs in both its active liganded and inactive unliganded states, and structural information of the EpoR transmembrane domain will be crucial for understanding the molecular details of EpoR activation. However, as yet there is no molecular structure of any cytokine receptor transmembrane or intracellular domain, and we do not know how phosphorylation of the appended JAK2s are triggered by cytokine binding. To approach this problem, the EpoR
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transmembrane domain has been fused to a leucine zipper coiled-coil segment from the Put3 transcription factor, and active and inactive orientations of EpoR transmembrane dimer have been deduced by changing the dimer interface through serial deletions in the transmembrane domain of the chimeras (6). In these chimeras, the parallel dimeric helices dictated by the Put3 coiled-coil domains most likely resemble the symmetric activated configuration induced by the Epo mimetic peptide, rather than the asymmetric active conformation triggered by Epo binding (6).

Here we undertook a less invasive way to investigate the active states of the EpoR. Because the EpoR transmembrane and juxtamembrane domains form a rigid helix that locks it in an inactive configuration in the absence of ligand, mutations in this region may mimic the conformational changes that the Epo binding imposes on the EpoR, causing the realignment of the intracellular domains and the associated JAK2 kinases, and leading to activation of JAK2. By comprehensively screening designed libraries of EpoRs with all possible single and many multiple mutations in their transmembrane domains, we identified several mutations that activate EpoR in the absence of Epo. Many introduce charged amino acids in the transmembrane domain, but several have changes of either of the first two transmembrane domain residues (Leu253 and Ile257) to cysteine. We then performed cysteine-scanning mutagenesis of the EpoR juxtamembrane extracellular and transmembrane domains. Many mutants form disulfide-linked dimers, but only EpoR dimers linked by cysteine at positions 223, 226, or 227 activate all of the EpoR signal transduction pathways we tested and also support proliferation of Ba/F3 cells in the absence of cytokines. These results suggest that activation of dimeric EpoR by Epo binding is achieved by reorienting the EpoR transmembrane domains, and that certain disulfide-bonded dimers represent the activated dimeric conformation of the EpoR. Structural analysis of disulfide-linked synthetic EpoR transmembrane domains, locked in either an inactive or an active state, as defined by these mutations, will elucidate how the conformational changes in a dimeric EpoR activates JAK2 and downstream signaling pathways.

EXPERIMENTAL PROCEDURES

Mutant Library Construction—The murine EpoR coding region was inserted in a bicistronic retroviral vector to generate pMX-mEpoR-IRES-GFP vector (13). Silent mutations were made to create 4 unique restriction sites around the EpoR transmembrane and juxtamembrane IRES-GFP vector (13). Silent mutations were made to create 4 unique inserted in a bicistronic retroviral vector to generate pMX-mEpoR-the essential Leu253, and 19 retroviral libraries expressing these mutant (NNS) in tandem (Fig. 1A). The random codons encompass the entirety of the transmembrane and juxtamembrane regions (226–256) except the essential Leu253, and 19 retroviral libraries expressing these mutant EpoRs were generated. Each library has a complexity from 1,000 (2 random codons) to 32,000 (3 random codons). IL-3-dependent Ba/F3 cells were infected with these libraries. To avoid multiple infections in one cell, the infection efficiency was controlled so that only 10% of Ba/F3 cells were infected with retroviruses. Green fluorescent protein (GFP) positive cells were isolated by FACS after 48 h. These cells were then plated in RPMI 1640 medium containing 10% fetal bovine serum without IL-3 and Epo. Surviving clones were selected after 7–10 days of culture. The identities of these clones were revealed by genomic PCR with vector-specific primers and subsequent sequencing of the PCR products (Fig. 1C). Assay for Epo-independent and Epo-dependent Proliferation—Ba/F3 cells expressing wild-type or mutant EpoRs were extensively washed and plated in RPMI medium with 10% FBS, without adding any growth factors. Cell numbers were counted at 24, 72, and 120 h with a Coulter particle counter.

To measure Epo-dependent proliferation, Ba/F3 cells expressing wild-type or mutant EpoRs were lysed in Nonidet P-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris, pH 7.4) with sodium vanadate and protease inhibitors (Roche Diagnostics). These lysates were then incubated with antibodies against JAK2 (Upstate Biotechnology) or STAT5 (Santa Cruz Biotechnology) as indicated. Immune complexes were recovered by adding protein A-Sepharose beads (Amersham Biosciences). Protein A-Sepharose beads were then washed extensively in lysis buffer, and the proteins associated with the beads were eluted by boiling in SDS sample buffer. Samples were separated on a 7% Tris acetate NuPAGE gel from Invitrogen, and transferred to nitrocellulose membranes (Schleicher & Schuell). These membranes were probed with antibodies specific for phosphotyrosine (4G10, Upstate Biotechnology). Bound antibodies were detected by incubation with horseradish peroxidase-coupled secondary antibodies (anti-mouse, 1:5000, Jackson ImmunoResearch Laboratories) and the enhanced chemiluminescence system (Pierce).

To reprobe the immunoblots for total JAK2 and STAT5, nitrocellulose membranes were incubated in 62.5 mM Tris (pH 6.8) with 0.1 M β-mercaptoethanol and 2% SDS for 30 min at 50 °C, then washed extensively in 10 mM Tris (pH 8) with 150 mM NaCl and 0.2% Tween 20. The membranes were then reprobed with antibodies specific for JAK2 (Upstate Biotechnology) or STAT5 (Santa Cruz Biotechnology) as described.

Measurement of Surface EpoR by Equilibrium Epo Binding—Purified carrier-free recombinant mouse Epo (R&D Systems) was labeled with iodine-125 (PerkinElmer Life Sciences) using IODO-GEN (Pierce). Ba/F3 cells expressing wild-type or mutant EpoRs from a bicistronic vector also expressing GFP were first gated for their GFP intensity, and cells with similar GFP expression were sorted. These cells were then incubated in pH 7.3 culture medium with different concentrations of 125I-Epo for 18 h at 4 °C, in duplicate. Bound and free 125I-ligand was separated by centrifuging cells through a layer of PBS. Parental Ba/F3 cells were used as a control to measure nonspecific Epo binding. Bmax was determined by fitting the model for a single-class of non-cooperative binding sites to data obtained over a range of ligand concentrations, using MATLAB software (The Mathworks, Inc., Natick, MA), and the total number of receptors on the cell surface was calculated using Bmax Avogadro number, and the number of cells assayed (14).

Endo H Digestion of the EpoR—Ba/F3 cells stably expressing wild-type or mutant EpoRs were lysed in Nonidet P-40 lysis buffer with protease inhibitors at 4 °C. Proteins in the cell lysates were denatured in 0.5% SDS and 1% β-mercaptoethanol followed by digestion with endoglycosidase H (Endo H) (New England Biolabs, 500 units) at 37 °C for 16 h. The digested products were then separated on SDS-PAGE, transferred to nitrocellulose membrane, and Western blotted with anti-EpoR antibodies (M-20, Santa Cruz).
Mutations in the Transmembrane Region of EpoR Result in Epo-independent Proliferation of Ba/F3 Cells

Results

Mutations in the Transmembrane Region of EpoR Result in Epo-independent Proliferation of Ba/F3 Cells—EpoR transmembrane and juxtamembrane domains play critical roles in receptor dimerization and activation (5, 12). We hypothesized that certain mutations in this region could mimic the activated dimeric state of EpoR, thus supporting the survival and proliferation of erythroid progenitors in the absence of Epo. A few activating mutations in the transmembrane and juxtamembrane domain have been identified in other cytokine receptors (15–17), but there has been no systematic attempt to identify them in EpoR.

To create a comprehensive set of EpoR mutant libraries, we undertook a saturation mutagenesis approach detailed under “Experimental Procedures.” Briefly, we used synthesized oligonucleotides with two or three random codons in tandem to replace the coding sequences for the EpoR transmembrane and juxtamembrane domains in the bicistronic pMX-mEpoR-IRES-GFP vector also encoding GFP (13) (Fig. 1A). In total 19 retroviral libraries expressing these mutant EpoRs were generated and used to infect IL-3-dependent Ba/F3 cells. Ba/F3 cells expressing mutant EpoRs were sorted by FACS, and ligand-independent Ba/F3 clones were isolated after 7–10 days in the absence of Epo and IL-3. Their EpoR coding regions were sequenced following genomic PCR (Fig. 1C). These mutant EpoRs were then reconstructed and expressed individually in Ba/F3 cells using a bicistronic retrovirus in which GFP is co-expressed from the same mRNA as the EpoR. Stable cell lines expressing these mutant receptors were sorted for the same level of GFP fluorescence and then tested for their ability to support Epo-dependent and Epo-independent growth (Fig. 2). The confirmed mutations that constitutively activate the EpoR are shown in Table 1.

Most mutations reside in the very N-terminal 2 or 3 residues of the transmembrane domain, which predominately are mutated to polar residues or cysteines. These mutations support Ba/F3 cells in the absence of Epo, as shown in Fig. 2. Compared with Ba/F3 cells bearing the wild-type EpoR, which undergo apoptosis in the absence of Epo, cells that express these mutant receptors continue to survive and proliferate at various rates (Fig. 2A). Simply deleting one, two, or three residues from the very N terminus of the transmembrane domain does...
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not cause Epo-independent receptor activation or cell proliferation, suggesting that constitutive cell proliferation in these mutants is not caused by changing the length of the hydrophobic region of the EpoR transmembrane domain (data not shown). Fig. 2B shows that cells expressing one of these constitutive mutants, L226N/I227K/L228K, grew at a faster rate in the presence of physiological concentrations of Epo (0.01–0.1 units/ml). In contrast, cells expressing the constitutively active mutants L226C/I227C and L236W/I237K showed very little Epo-stimulated growth over the same range of Epo concentrations.

Fig. 2C, lanes 4–9, shows that Ba/F3 cells expressing the constitutively active EpoR mutants L226C, I227C, L226C/I227C, L226N/I227K/L228K, L226V/I227P, and L236W/I237K all exhibit activation of JAK2 even when deprived of growth factors for 4 h. In contrast and as expected no activation of JAK2 was seen when Ba/F3 cells expressing the wild-type EpoR (Ba/F3-EpoR cells) were deprived of Epo and IL-3 for 4 h (lane 2). The level of constitutive JAK2 activation by these mutant EpoRs is comparable with that seen in Ba/F3 cells expressing the wild-type EpoR but growing continuously in a physiological concentration of Epo, about 0.1 unit/ml (lane 3). This level of JAK2 activation is, as expected, less than that seen in Ba/F3 cells expressing the wild-type EpoR deprived of Epo for 4 h and then stimulated with a high concentration of Epo (10 units/ml) for 10 min (lane 1). In cells expressing the wild-type EpoR growing continuously in the presence of Epo a number of negative regulatory proteins are induced or activated, dampening the signaling from the Epo-activated EpoRs. Thus Fig. 2C documents that in Ba/F3 cells expressing any of the constitutively active mutant EpoRs, there was an expected level of JAK2 phosphorylation in the absence of Epo stimulation. Thus these receptor mutants assume an active conformation even in the absence of Epo that is permissive for JAK2 trans-phosphorylation.

Notably, mutation of either of the two most exoplasmic residues of the EpoR transmembrane domain, Leu226 or Ile227, to cysteine caused Epo-independent growth and constitutive JAK2 phosphorylation in Ba/F3 cells (Fig. 2C and Table 1). Considering the unique property of cysteine residues, we suspected that the constitutive activation of these EpoRs was a result of disulfide bond formation that fixed the EpoR dimers in an activating conformation. Three other mutations in the murine EpoR extracellular domain, R129C, E132C, and E133C, also form disulfide-linked constitutively active EpoR homodimers, suggesting that these mutant receptors are also "locked" in an active dimeric conformation (4, 18, 19). If indeed disulfide formation in these mutants lead to their constitutive activation, unlike the R129C mutation in the extracellular domain, the structure of these disulfide-dimerized EpoR transmembrane domains could provide structural information about the active orientation of the EpoR transmembrane domain and intracellular domains.

Cysteine Scanning Mutagenesis of EpoR Transmembrane and Juxtamembrane Domains—To further study the effect of cysteine substitution in the EpoR transmembrane and juxtamembrane domains, we carried out cysteine scanning mutagenesis. We changed each of the transmembrane residues (amino acids 226–247) one-at-a-time to cysteine. We also mutated adjacent residues in the exoplasmic and cytosolic juxtamembrane domains (residues Asp222 to Pro225, and Ser246 and His249, respectively) to cysteine because the exoplasmic juxtamembrane domain was not included in our original saturation mutagenesis. These mutant EpoRs were introduced into the bicistronic pMX-IRE-GFP vector and expressed in populations of Ba/F3 cells through retroviral infection. EpoR-expressing cells were isolated by FACS sorting on the basis of GFP fluorescence (13). These cells were then plated in the RPMI medium with 10% serum, and their cytokine-independent growth was measured. Ba/F3-EpoR cells growing in the presence of 0.1 unit/ml Epo were used as controls.

Of these mutants, only mutants L223C in the exoplasmic domain and only L226C and I227C in the transmembrane domain supported exen-
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TABLE 1
Constitutively activating mutations in the EpoR transmembrane domain identified by a random mutagenesis screen

| Mutant | Amino Acid | Description |
|--------|------------|-------------|
| L226C | NKK        | Dimerization |
| L227C | NRE        | Dimerization |
| L228C | NTH        | Dimerization |
| L229C | VP         | Dimerization |
| L230C | CC         | Dimerization |
| L231C | C          | Dimerization |
| L232C | CA         | Dimerization |
| L233C | WC         | Dimerization |

* Amino acid sequences from EpoR transmembrane domain (in bold) and juxtamembrane domain.

** Amino acids that are identified to wild-type EpoR are represented by a dot. Only mutated residues are shown using the one-letter abbreviation.

sive ligand-independent growth of Ba/F3 cells (Fig. 3, A and B). Another mutation, D224C, supported a much weaker ligand-independent proliferation of Ba/F3 cells; following long times in cytokine-free culture a small fraction of the cells survived and grew poorly, whereas the majority of the cells died. Thus the D224C EpoR mutant can support a low level of receptor self-activation that prevents some Ba/F3 cells from undergoing apoptosis. In contrast, replacement of amino acid residues 228–249 with cysteine yields receptors with wild-type properties. Ba/F3 cells expressing these receptors responded normally to Epo stimulation and did not exhibit any Epo-independent proliferation (Fig. 3 and data not shown). This suggests that our original saturation mutagenesis and screening did not miss any other activating cysteine substitutions in the targeted EpoR segments.

Fig. 4A shows that Ba/F3 cells expressing EpoRs with cysteine substitutions at amino acids 222–228 all proliferate in the presence of 0.1 unit/ml Epo, as judged by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (see “Experimental Procedures”). Only D224C and P225C showed an impaired response to Epo, whereas other mutants responded normally to Epo (D222C, L226C, I227C, L228C) or exhibited hypersensitivity to 0.1 unit/ml Epo (L223C).

Formation of Disulfide-linked Receptor Dimers Is Necessary but Not Sufficient for Epo-independent Activation—Having found that cysteine substitutions at Leu(223), Leu(226), and Ile(227) constitutively activate the EpoR, we suspected that formation of intermolecular disulfide bonds at these positions might drive receptor self-activation. To determine whether these mutants, when expressed in Ba/F3 cells, indeed form intermolecular disulfide bonds, non-reducing SDS-PAGE was performed (Fig. 4B). In contrast to wild-type EpoR, EpoR mutants with cysteines at positions 222–228 all formed disulfide-linked dimers. Dimeric forms of EpoR could not be detected in mutants with cysteine substitution at or following residue Thr(229) (T229C to H249C; Fig. 3D and data not shown). This probably results from the inaccessibility of these cysteine residues to the endoplasmic reticulum (ER) luminal enzymes that catalyze disulfide bond formation. These mutants also do not exhibit constitutive activation in Ba/F3 cells, suggesting that in these EpoR mutants with cysteine substitutions formation of an intermolecular disulfide bond is necessary for Epo-independent activation.

Importantly, the data in Fig. 4B shows that formation of an intermolecular disulfide bond is not sufficient for Epo-independent activation; clearly the level of the disulfide-linked dimer is not correlated with the extent of Epo-independent proliferation. As an example, as judged by the ratio of dimeric to monomeric EpoR (Fig. 4B), D224C shows the strongest extent of dimerization, with almost 40% of total cellular EpoR in a disulfide-linked dimer. This mutant exhibits very low levels of Epo-independent proliferation (Fig. 4A). Mutant D222C, which forms the next highest proportion of disulfide-linked dimers, does not support ligand-independent proliferation in Ba/F3 cells. The extent of formation of L223C disulfide-linked dimers is much lower than that of either D224C or D222C, yet L223C supported the strongest Epo-independent cell proliferation among this group. Low and approximately equal levels of disulfide-linked dimers were formed by the P225C, L226C, I227C, and L228C mutants, yet of these only L226C and I227C supported Epo-independent cell growth. These results suggest that receptor activation is not accomplished simply by bringing two receptors into close proximity, as suggested by Livnah et al. (20).

All Constitutively Active EpoR Cysteine Mutants Except D224C Undergo Normal Biosynthesis and Have Normal Surface Expression—In all our studies wild-type and mutant EpoRs are expressed in Ba/F3 cells using a bicistronic retroviral vector in which expression of GFP, promoted by the IRES sequence, occurs from the same mRNA as the EpoR (13). Infected cells were sorted for the same levels of GFP expression,
and thus we anticipated that the same amount of EpoR, whether wild-type or mutant, would be expressed in these uncloned cell populations. Indeed Fig. 5A shows that this is the case, with the exception that the amount of D224C and P225C EpoR is slightly elevated.

Only a small portion of newly synthesized EpoRs reach the cell surface; in transfected Ba/F3 cells most remain in the ER as an Endo H-sensitive form and are degraded (21, 22). Importantly, Fig. 5A shows that cells expressing mutants D222C, L223C, L226C, and I227C all show approximately the same amounts of the 66-kDa Golgi-processed Endo H-resistant EpoR and 64-kDa endoplasmic reticulum localized Endo H-sensitive EpoR forms as do cells expressing the wild-type EpoR. This suggests that these four mutant Epo receptors undergo normal biosynthetic processing to the Golgi and also normal receptor turnover. This conclusion is corroborated by the data in Fig. 5, B and C, that shows that cells expressing EpoR mutants D222C, L223C, L226C, and I227C all show approximately the same amount of cell surface Epo receptors, ~1300 Epo molecules bound per cell, similar to cells expressing the wild-type EpoR.

Cells expressing EpoR mutants P225C and especially the weakly constitutively active D224C exhibit defects in receptor biosynthesis. As shown in Fig. 5A Ba/F3 cells expressing EpoR D224C and also P225C have much more 64-kDa endoplasmic reticulum localized Endo H-sensitive EpoR. This indicates that a larger fraction than normal of EpoRs P225C and D224C are retained in the endoplasmic reticulum, presum-
Activated in Ba/F3 Cells Expressing Activated EpoR Mutants panels
rocine antibody PY99, and analyzed by Western blotting using an anti-EpoR antibody.
ure EpoR phosphorylation, cell lysates were immunoprecipitated with anti-phosphoty-
then stimulated (IL-3 and Epo for 4 h. The same number of starved wild-type EpoR expressing cells were
evated in Ba/F3 cells expressing HA-tagged wild-type or mutant EpoRs were deprived of
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EpoR Downstream Signal Transduction Pathways Are Constitutively activated in Ba/F3 cells expressing EpoR mutants L223C, L226C, and I227C. Twenty-five million Ba/F3 cells expressing HA-tagged wild-type or mutant EpoRs were deprived of IL-3 and Epo for 4 h. The same number of starved wild-type EpoR expressing cells were then stimulated (WT+) or not (WT−) with 10 units/ml Epo for 10 min. Cells were lysed and immunoprecipitated with antibodies specific for JAK2 (A) or STAT5 (B), and then analyzed by Western blotting using anti-phosphotyrosine antibody 4G10 (A and B, upper panels), or using antibodies against JAK2 and STAT5 (A and B, bottom panels). C, to measure Epo phosphorylation, cell lysates were immunoprecipitated with anti-phosphotyrosine antibody PY99, and analyzed by Western blotting using an anti-EpoR antibody.

ably because a higher fraction than normal fail to be folded properly. These mutants may also be degraded at a slower than normal rate, as is the case for the R129C EpoR mutant (18). In addition, the highly disul-
fide-linked D224C mutant has much less 66-kDa Golgi-processed Endo H-resistant EpoR than do cells expressing wild-type EpoR. This conclusion is corroborated by the data in Fig. 5, B and C, that show that cells expressing EpoR mutant D224C have many fewer cell surface Epo receptors, ~700 Epo molecules bound per cell, than do cells expressing the wild-type EpoR. We obtained similar results by FACS analyses measuring surface HA-tagged EpoRs (data not shown).

Taken together, these data indicate that mutants D222C, L223C, L226C, and I227C are processed normally through the Golgi and appear normally on the cell surface. The weakly activated mutant D224C may be folded less efficiently than wild-type EpoR in the ER, as less Golgi-processed receptor is formed and consequently less appears on the cell surface. This decrease in numbers of surface D224C molecules may contribute to its low Epo-dependent growth rate when expressed in Ba/F3 cells (Fig. 4A).

EpoR Downstream Signal Transduction Pathways Are Constitutively Activated in Ba/F3 Cells Expressing Activated EpoR Mutants—The data in Fig. 6 shows that three principal signal transduction proteins are activated by phosphorylation in cells expressing EpoR mutants L223C, L226C, and I227C cultured in the absence of Epo; these are the same mutants that support Epo-independent growth of Ba/F3 cells. As controls for these studies, Ba/F3-EpoR cells were starved of Epo and then treated with Epo for 10 min, the time the peak activation of signaling is achieved. Robust Epo-induced tyrosine phosphorylation of JAK2 (panel A), STAT-5 (panel B), and the EpoR itself (panel C) was seen. For cells expressing EpoR mutants D222C, L223C, D224C, P225C, L226C, and I227C, phosphorylation of these signaling proteins was measured during culture in the absence of Epo (or IL-3) stimulation. As discussed above in the context of Fig. 2C, activation of signaling proteins is expected to be much lower than in starved cells stimulated by high concentrations of Epo, because during steady-state several cell growth negatively acting signaling proteins, such as SHP-1 and SOCS proteins, become activated (23, 24). Indeed Fig. 6A shows that cells expressing EpoR D223C, D224C, L226C, and I227C exhibit constitutive tyrosine phosphorylation of the JAK2 protein, as detected by immunoprecipitation with anti-JAK2 rabbit IgG and Western blotting with the 4G10 antibody. No phosphorylation of JAK2 was evident in cells expressing wild-type EpoR, D222C or P225C mutants when cultured in the absence of Epo. As a control, all cells contained a similar amount of total JAK2 protein (Fig. 6A, lower panel). Thus tyrosine phosphorylation of JAK2 correlates with the ability of mutant EpoRs to support Epo-independent cell proliferation.

Similarly, as is shown in panel B, in the absence of Epo, Ba/F3 cells expressing wild-type EpoR, or mutants D222C or P225C showed only background levels of STAT5 phosphorylation. In contrast, cells expressing EpoR mutants L223C, D224C, L226C, or I227C showed robust levels of STAT5 phosphorylation. All cells contained the same amounts of total STAT5 protein (Fig. 6B, lower panel).

Tyrosine phosphorylation of the EpoR cytosolic domain creates docking sites for many signaling proteins, and is essential for normal activation of STAT5, phosphatidylinositol 3-kinase, mitogen-activated protein kinase, and also negative regulators such as SHP-1 and SOCS proteins (23–25). Fig. 6C shows that cells expressing EpoR mutants L223C, D224C, L226C, or I227C and cultured in the absence of Epo also exhibit robust tyrosine phosphorylation of the EpoR itself, in contrast to cells expressing wild-type EpoR, D222C or P225C mutants when cultured in the absence of Epo. As is shown in panel C, the constitutive phosphorylation of JAK2 and STAT5 in these cells may be mediated mainly by D224C mutants that are trapped in the ER. In cells expressing D224C mutant EpoR, the insufficient processing and surface expression of D224C EpoR (Fig. 5) may contribute to the low level of phosphorylated surface EpoR, and in turn affect its ability in supporting factor-independent growth, because of defects in certain signaling pathways that are dependent on surface EpoR.

Overall, these results demonstrate that mutants L223C, L226C, and I227C indeed are active in the absence of Epo, as they support activation by phosphorylation of three major signaling proteins normally activated by Epo addition, JAK2, the EpoR itself, and STAT5. We expect that these mutant EpoRs also activate the phosphatidylinositol 3’-kinase and Ras-MAP kinase pathways although we have not explicitly studied this.

**DISCUSSION**

Here we performed saturation mutagenesis of the EpoR transmembrane and juxtamembrane domains and identified constitutively activated receptor mutations by selecting mutant EpoRs that support Epo-independent proliferation of cytokine-dependent Ba/F3 cells. We
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Our studies focused mainly on the role of the EpoR transmembrane domain and intracellular juxtamembrane domain; the entire region we studied contains 30 amino acids. Thus we were able to take a comprehensive strategy in creating our mutant libraries. By using annealed oligonucleotides containing random codons we were able to generate mutants with all possible 20 amino acids at each codon. To obtain mutants with more drastic impacts on the structure of EpoR transmembrane domain and juxtamembrane domain than point mutations, we grouped every two or three adjacent codons together and substituted all of them with random codons. This enabled us to create multiple mutations that could alter the receptor more effectively.

Using this strategy, we did isolate several mutants that are constitutively active and that contained two or more mutations (Table 1). In particular we isolated three constitutively active triple mutants, each of which introduced one or more polar residues within the extracellular part of the transmembrane domain. We have dissected the contribution of individual point mutations in the constitutively active double mutants L226V/I227P and L236W/I237K. We found that only the I227P EpoR mutant supports weak factor-independent growth, and that the single mutants L226V, L236W, and I237K are unable to support constitutive growth of Ba/F3 cells (data not shown). It appears that both point mutations are needed to generate a sufficient structural alteration to activate EpoR signaling. Structures of these double mutants compared with that of wild-type EpoR will shed light on the activation of the receptor.

Inter-molecular Disulfide Bond Formation and EpoR Activation—In our cysteine scanning mutagenesis, residues across the transmembrane domain, from Asp222 to His249, were replaced individually with cysteines. We detected inter-molecular disulfide formation between two dimerized EpoR molecules expressed in Ba/F3 cells by non-reducing SDS-PAGE; only EpoRs with cysteines N-terminal to position 229 could form disulfide bonds. Mutant D224C formed the highest percentage of dimers, followed in order by D222C, L223C, and P225C. L226C, I227C, and L228C each exhibited a very small fraction of molecules in a dimeric form. Mutants with cysteines introduced at positions 229–256, which are either completely buried in the membrane or at the cytosolic surface, did not form disulfide-linked dimers, apparently because these residues are not exposed to proteins such as protein-disulfide isomerase that catalyze disulfide bond formation in the ER lumen. Similar observations have been made in other cysteine scanning mutagenesis protocols, in that only the first 3 or 4 residues in the transmembrane domain are able to form disulfide bonds (26).

Strikingly, the extent of formation of intermolecular disulfide bonds was not correlated with the extent of Epo-independent proliferation. EpoR D224C showed the strongest extent of dimerization but supported very low levels of Epo-independent proliferation. However, the case for D224C is complicated by the fact that a larger than normal fraction of newly made D224C EpoR is retained in the ER and eventually degraded, whereas a smaller than normal fraction reaches the cell surface. However, the extent of formation of L223C disulfide-linked dimers was much lower than that of D222C, yet L223C supported extensive Epo-independent cell proliferation, and D222C did not. Similar low levels of disulfide-linked dimers were formed by the P225C, L226C, I227C, and L228C mutants, yet of these only L226C and I227C supported Epo-independent cell growth. In all cases constitutive phosphorylation and thus activation of JAK2, EpoR, and STAT5 in cells expressing these mutants correlated with their ability to grow in the absence of cytokines.

Thus our results establish that receptor activation is not simply accomplished by bringing two receptors into close proximity through
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A recent independent study by Kubatzyk et al. (28) used cysteine scanning mutagenesis to identify the same three constitutively active mutants: L223C, L226C, and I227C. Chemical cross-linking studies were interpreted as showing that the juxtamembrane-transmembrane junctional segment encompassing amino acids Leu225 through Leu230 is non-helical, and their molecular dynamics and NMR studies indicated that this junction forms an N-terminal helix cap. However, the exact structure of the junctional region (residues Asp222 to Leu230) is uncertain as is their role in maintaining the active and inactive conformations of EpoR. Defining these structures is an important area for further studies.

Proper Orientation of the Two EpoR Transmembrane Domains Is Essential for JAK2 Activation—Activation requires the function of the JAK2 kinase associated with the membrane-proximal segment of the EpoR intracellular domain. JAK2 is comprised of 7 JAK homology (JH) domains, named JH1 to JH7. In isolation the C-terminal JH1 domain is a catalytically active tyrosine kinase. The adjacent JH2 domain is a pseudokinase domain that suppresses the basal kinase activity of JAK2 JH1. Deleting the JH2 domain relieves its inhibition of JH1 kinase activity, leading to a constitutively active JAK2 (29). The JH2 domain weakly associates with JH1, and the JH1 domain preferentially interacts with

Asp222 is at the very end of the D2 domain, that this junction forms an N-terminal helix cap. However, the exact structure of the junctional region (residues Asp222 to Leu230) is uncertain as is their role in maintaining the active and inactive conformations of EpoR. Defining these structures is an important area for further studies.

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another JH1 domain, possibly leading to its activation (30). Thus JAK2 activation might result from a relative movement in the two EpoR intracellular domains of a homodimeric surface receptor, juxtaposing the two appended JAK2 molecules in such an orientation that allows activating intermolecular JH1–JH1 interactions instead of the weaker inhibitory intramolecular JH1–JH2 interaction. If this is the case, there should be more that one conformation of two EpoRs that can activate JAK2. Indeed, the crystal structures of Epo-EBP and EMP-EBP complexes show some very significant differences. Epo, the most potent ligand for EpoR, is an asymmetric molecule, which positions the two EpoR extracellular domains asymmetrically with a 120° angle between the two D1 domains (7). The symmetric EMP dimer positions the EpoR extracellular domains symmetrically with a 180° angle between them (8). These two conformations may very likely generate different orientations of the intracellular domains. Although less efficient than Epo, EMP can activate EpoR signaling. Also, symmetrically rotating the EpoR transmembrane domain and intracellular domain by deletions in the Put3-EpoR chimeras can generate constitutively activated receptors (6).

Our view of possible EpoR activation is summarized in Fig. 7. Inactive EpoRs exist as symmetric dimers on the cell surface. The hatched areas in the transmembrane domain represent the interacting surface in the inactive EpoR dimer, involving the same set of residues in the two subunits (Fig. 7A). When a residue in the junction region of the EpoR extracellular domain and transmembrane domain is mutated to cysteine, an intermolecular disulfide bond may form. If the disulfide bond is formed at or near the dimer interface, it will be accommodated with little structural alteration and the receptor remains inactive (Fig. 7B). If the disulfide bond falls outside of the dimer interface, the transmembrane domains must change their relative orientation. The final product can have a symmetrical arrangement, like the activated conformation of the Put3-EpoR chimera (6), through symmetric rotation that brings the appended JAK2s into close proximity (Fig. 7C). Certain disulfide bonds are likely to generate an asymmetrical configuration, similar to that of the Epo-stimulated EpoR dimer (7). In this case, two different sets of residues from each EpoR monomer will be at the dimer interface of the transmembrane domain as a result of an asymmetric rotation (Fig. 7D). Exactly which conformation the mutant receptor will take likely depends on many other factors. Considering the strong interactions in the EpoR transmembrane dimer (9), we think the model shown in Fig. 7D is a more likely choice for the disulfide-linked mutants, because only this conformation can be achieved by a slight movement of the two dimerized EpoR. Because Epo is an asymmetrical molecule that upon binding positions the two EpoR extracellular D1 domains in a 120° angular configuration, the Epo-activated EpoR dimer may also assume a similar asymmetrical conformation (Fig. 7E). Structural analysis on wild-type and mutant peptides corresponding to these EpoR segments should provide us with a much needed answer.