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Homodimerization and constitutive activation of the erythropoietin receptor

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ABSTRACT **The erythropoietin receptor (EPO-R) is a member of the recently described cytokine receptor superfamily. A constitutively active (hormone independent) form of the EPO-R was isolated that has a single amino acid change in the exoplasmic domain, converting arginine-129 to cysteine (R129C). Since EPO-Rs containing R129S, R129E, and R129P mutations are functionally wild type, the presence of cysteine at residue 129, and not the loss of arginine, is required for constitutive activity. Several mutant forms of the EPO-R were analyzed; all constitutively active mutants form disulfide-linked homodimers, whereas EPO-responsive or inactive forms of the receptor do not. Monomers and disulfide-linked dimers of the constitutive receptor are present on the plasma membrane and bind EPO with a single affinity. Homodimerization of the EPO-R is likely to play a role in ligand-induced signal transduction, and disulfide-linked dimerization of the constitutive receptor may mimic this step.**

Erythropoietin (EPO) is a serum glycoprotein hormone required for the survival, proliferation, and differentiation of committed erythroid progenitor cells. The murine EPO receptor (EPO-R) cDNA was isolated by expression cloning (1) and was found to have sequence homology with other cytokine receptors (2). Conserved structural features of the cytokine receptor superfamily include four similarly spaced exoplasmic cysteine residues, as well as a motif, WSXWS, located in the exoplasmic domain close to the membrane-spanning region (3). The EPO-R and other members of the cytokine receptor family do not contain kinase-related or nucleotide-binding consensus sequences in their cytoplasmic domains and the intracellular signaling pathways they initiate after ligand binding have yet to be defined.

Although little is known of the mechanisms by which cytokine receptors transduce their signal, dimerization of the receptors is thought to play a role. The receptors for interleukins 2, 3, 5, and 6, as well as granulocyte–macrophage colony-stimulating factor, contain at least two different subunits (4–8), while the ligand binding subunits of the granulocyte colony-stimulating factor receptor, prolactin receptor, and growth hormone receptor form homodimers (9–11). Dimerization has been postulated to yield high-affinity receptors and also to provide the first step in the signal transduction pathway (11, 12).

Expression of the cloned EPO-R cDNA in the interleukin 3-dependent pro-B-cell line BA/F3 allows the cells to grow in response to EPO, demonstrating that the EPO-R can functionally transmit a growth signal (13). The recent demonstration that the mutation of arginine-129 to cysteine (R129C) results in a constitutively active (14) and oncogenic form (15) of the EPO-R is provocative in that it implicates the formation of aberrant inter- or intramolecular disulfide bonds in the process of receptor activation.

The role of the new cysteine residue in the constitutively active receptor and the possibility that this receptor may have an altered disulfide-bonding pattern were investigated by both biochemical and mutagenesis approaches. The presence of cysteine at residue 129 is required for EPO-independent signaling. Analysis of several mutants of the EPO-R has revealed that all constitutively active mutants, but not the wild-type receptor or EPO-dependent mutants, form disulfide-linked homodimers in the endoplasmic reticulum (ER) and a fraction of these dimers are transported to the plasma membrane.

MATERIALS AND METHODS

Mutagenesis Techniques. The constitutive mutant of the EPO-R (R129C), the truncated form of the receptor (tEPO-R), and the constitutive, truncated form of the receptor (tEPO-R/R129C) were isolated by a retroviral transduction system (14). The remaining mutant EPO-Rs (see Fig. 1) were generated by polymerase chain reaction, with synthetic oligonucleotide primers encoding the desired amino acid substitutions. Mutant EPO-R cDNAs were subcloned into the mammalian expression vector pXM (16) and into M13mp18 and M13mp19 vectors. Sequences of the mutant cDNAs were confirmed by the dideoxynucleotide chain-terminating method, using synthetic oligonucleotides as primers.

Cell Culture Conditions and Transfections. The wild-type and mutant EPO-R cDNAs in pXM were introduced into BA/F3 cells by electroporation, and stable transformants were cloned as described (14). Interleukin 3-dependent, EPO-dependent, and factor-independent clones of BA/F3 cells were maintained as described (14).

Metabolic Labeling and Immunoprecipitation. BA/F3 cell lines expressing wild-type or mutant EPO-Rs were metabolically labeled with [^{35}S]methionine and cysteine (^{35}S-Express; NEN). Cell lysates were prepared in buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris·HCl (pH 7.4), 200 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride. Lysates were incubated with anti-peptide antibodies raised against N- or C-terminal peptides of the EPO-R (17), followed by incubation with protein A-agarose beads (Boehringer Mannheim). Proteins were eluted in gel sample buffer [1% SDS/10% (vol/vol) glycerol/80 mM Tris·HCl, pH 6.8] with or without 1% 2-mercaptoethanol.

Gel Electrophoresis and Immunoblot Analysis. One- and two-dimensional gel electrophoresis was carried out on SDS/7.5% polyacrylamide gels (18). For two-dimensional gels, polypeptides were separated first under nonreducing condi-

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Abbreviations: EPO, erythropoietin; EPO-R, EPO receptor; ER, endoplasmic reticulum; t-EPO-R, truncated form of EPO-R; bEPO, biotinylated EPO.

tions, and then after reduction with 5% 2-mercaptoethanol. Immunoblotting was performed as described (19). The filters were incubated with antisera raised to EPO-R peptides, followed by ^{125}I-labeled protein A (Amersham).

Scatchard Analysis and Biotinylated-EPO Binding. EPO was iodinated by the iodine monochloride method and had a specific activity of 4×10^6 cpm/pmol. Analyses of saturation binding were performed as described (20). Biotinylated EPO (bEPO) was prepared (21) and surface EPO-Rs were isolated as described, except that cross-linking of the bEPO–receptor complex was not performed (22).

RESULTS

The substitution R129C introduces a sixth cysteine residue into the EPO-R exoplasmic domain and confers constitutive (hormone independent) activity (Fig. 1, mutant R129C; ref. 14). Mutation of residue 129 to serine, glutamic acid, or proline yields a wild-type, not a constitutive, phenotype (data not shown). Since a cysteine residue at position 129, rather than the loss of an arginine, was crucial for constitutive activity, we suspected that the new cysteine may form an intramolecular disulfide bond, possibly with C179. To test this hypothesis, C179 was mutated to a serine residue either in the wild-type receptor (C179S) or in the constitutive mutant (R129C/C179S). Neither the EPO responsiveness of the wild-type receptor nor the constitutive activity of the R129C receptor was affected by this mutation (Fig. 1), demonstrating that C179 is not involved in EPO-induced activation of the wild-type receptor or in constitutive activation of the R129C mutant.

The possibility that R129C has other intramolecular disulfide bond rearrangements or has formed other intermolecular disulfide bonds remained. To determine whether the pattern of intramolecular or intermolecular disulfide bonding in R129C was different from that in the wild-type receptor, we immunoprecipitated metabolically labeled receptors and analyzed them by reducing and nonreducing SDS/PAGE. Both the newly synthesized wild-type EPO-R and R129C migrate with an apparent molecular mass of 64 kDa under reducing conditions (data not shown). Under nonreducing conditions, the newly synthesized wild-type EPO-R migrates with an apparent molecular mass of 64 kDa (Fig. 2, lane 4), while the constitutive receptor migrates as a monomer of ≈64 kDa as well as an oligomer of ≈160 kDa (lane 7). This oligomeric species accumulates during the 2-h chase at 18°C (lane 9) and it appears to be a disulfide-linked complex of the R129C receptors since it is not visible after reduction of the samples before SDS/PAGE (data not shown).

We assayed several mutants of the EPO-R for their ability to form disulfide-linked oligomers and found that all constitutively active mutants of the EPO-R form disulfide-linked oligomers, while all hormone-responsive or inactive forms of the receptor do not (Figs. 1 and 2). The constitutive R129C/C179S mutant forms disulfide-linked oligomers, although somewhat less efficiently than R129C (Fig. 2, lanes 13–15). tEPO-R/R129C also forms disulfide-linked oligomers that migrate faster than the R129C oligomers, as expected for an oligomeric species composed of truncated receptor molecules (lanes 19–21). tEPO-R and the C179S mutant, both of which are EPO responsive like the wild-type EPO-R, do not form detectable disulfide-linked oligomers (lanes 10–12 and 16–18).

To test further the correlation found between the presence of C129 in the EPO-R, disulfide-linked oligomerization, and constitutive activation, we assayed two inactive forms of the EPO-R (ws1 and ws1/R129C) for their ability to oligomerize. Both ws1 and ws1/R129C lack three residues, AWS, from the conserved WSXWS region and have a GA substitution in their place. In addition, the ws1/R129C mutant contains the R129C mutation. When expressed in BA/F3 cells, these mutant receptors are unable to transmit an EPO growth signal since the cells will only grow in the presence of interleukin 3 (Fig. 1). Both mutants are retained in the ER after synthesis and are likely to be misfolded (data not shown). Neither mutant receptor formed disulfide-linked oligomers (Fig. 2, lanes 22–27).

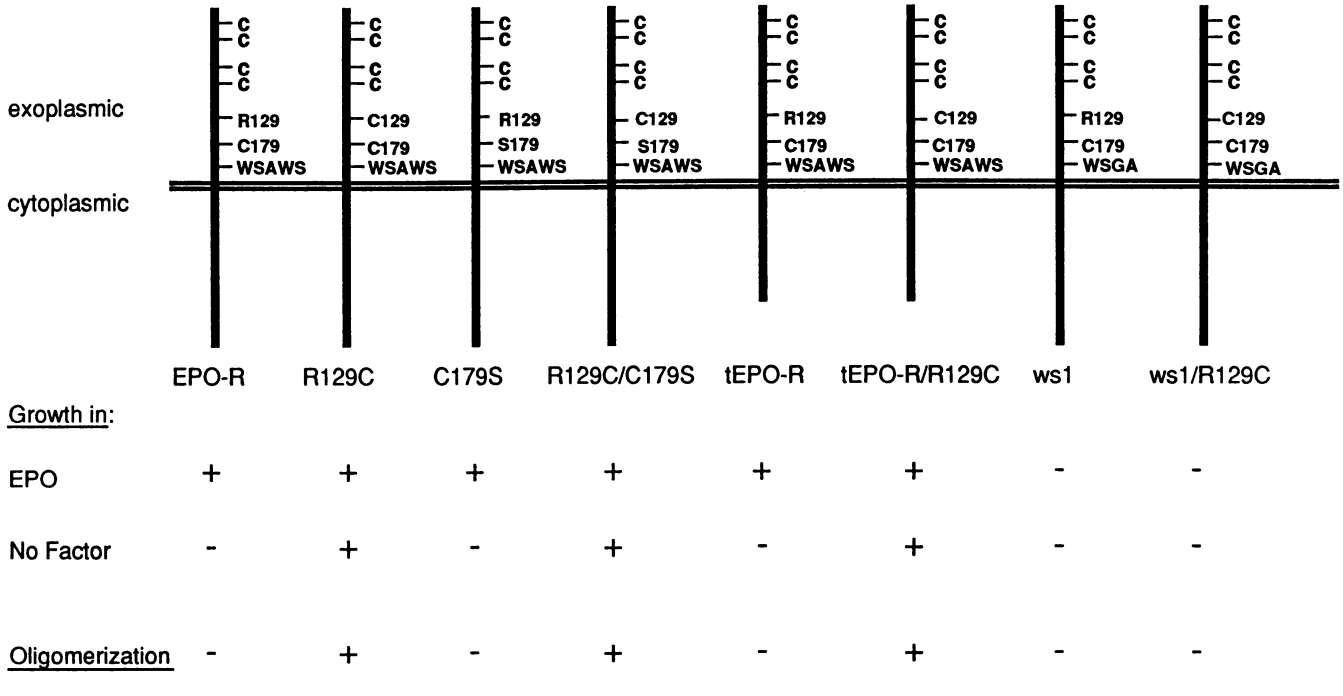

FIG. 1. Schematic diagram of mutant EPO-Rs. Mutants of the EPO-R cDNA were generated or isolated as described. The receptors were assayed for their ability to confer EPO-dependent or factor-independent growth in BA/F3 cells as described (13, 14). Assays for oligomerization were performed as indicated in the legend to Fig. 2. Residues 129 and 179 are indicated; the four conserved exoplasmic cysteine residues (C27, C37, C65, and C81) and the conserved WSAWS sequence (residues 207–211) are not numbered.

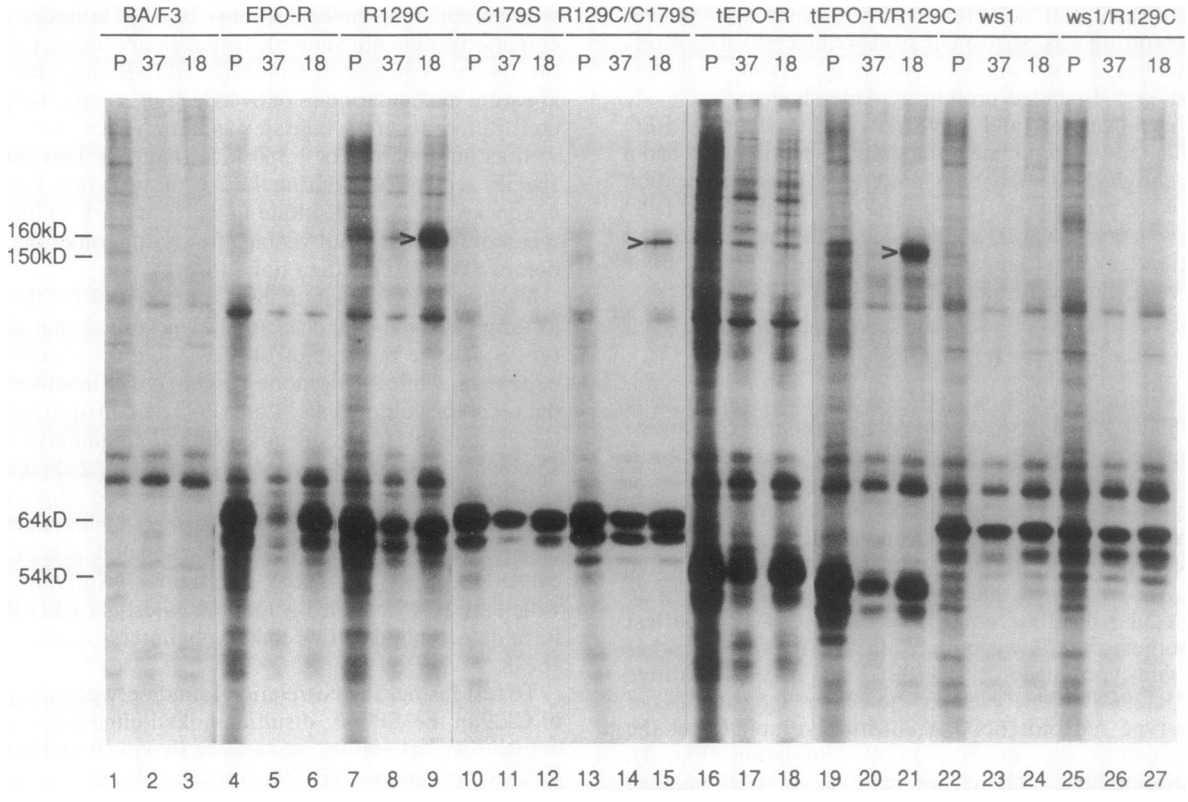

FIG. 2. Constitutively active mutants of the EPO-R form disulfide-linked oligomers. Parental BA/F3 cells or cells expressing wild-type or mutant EPO-Rs were pulse labeled (lanes P) with [^{35}S]methionine/cysteine for 10 min at 37°C and then chased for 2 h at 37°C (lanes 37) or 18°C (lanes 18). EPO-Rs were immunoprecipitated and separated by nonreducing gel electrophoresis. The migration positions of monomeric EPO-R (≈64 kDa) and monomeric, tEPO-R (≈54 kDa) are indicated on the left. The positions of oligomeric, constitutive EPO-R (≈160 kDa) and oligomeric, constitutive, tEPO-R (≈150 kDa) are indicated with arrowheads.

The identity of the polypeptides present in the EPO-R immunoprecipitates from parental BA/F3 cells and cells expressing the wild-type receptor, R129C, and tEPO-R/R129C, was determined by two-dimensional gel electrophoresis. The only polypeptides that were generated by reduction of the disulfide-linked oligomers were monomers of R129C (Fig. 3*C*) and tEPO-R/R129C (Fig. 3*D*), demonstrating that the disulfide-linked species are EPO-R homooligomers and eliminating the possibility that the constitutive EPO-R forms a heterooligomer with another polypeptide of similar size. The ability of R129C/C179S, but not the wild-type receptor or C179S, to form disulfide-linked oligomers (Fig. 2) suggests that only C129 is available for forming interchain disulfide bonds; thus, it is likely that the R129C oligomers are homodimers. The wild-type EPO-R migrated identically before and after reduction (Fig. 3*B*).

To determine whether the disulfide-linked homodimers are present on the cell surface, we used bEPO to isolate cell-surface receptors (22). Both R129C monomers and disulfide-linked dimers were found on the plasma membrane (Fig. 4, lane 3). Similarly, cells synthesizing R129C/C179S (lane 5) and tEPO-R/R129C (data not shown) expressed both monomers and dimers on the surface. After reduction, oligomers of the constitutive receptor were not detectable and only monomers were seen (lanes 8 and 10). Cells synthesizing hormone-responsive forms of the receptor (wild-type EPO-R, C179S, tEPO-R) did not express detectable levels of surface disulfide-linked dimers; as expected, only monomeric species are found on the plasma membrane (lanes 2 and 4; data not shown).

The presence of both monomers and dimers of R129C on the plasma membrane suggested that there may be two classes of surface receptors, perhaps corresponding to low- and high-affinity EPO-binding species. Scatchard analysis of ^{125}I-labeled EPO binding to BA/F3 cells expressing R129C demonstrates, however, that the surface receptors display a single affinity for EPO (K_d = 700 pM). Approximately 1000 surface receptors are expressed per cell (Fig. 5). Similarly, when EPO binding to the erythroid cell line HCD57 expressing R129C was examined, only a single class of receptors was detected even though both monomeric and dimeric forms of R129C were detected on the plasma membrane (data not shown). BA/F3 cells synthesizing wild-type EPO-R also displayed a single class of receptors (23).

DISCUSSION

The EPO-R can be activated by two different, hormone-independent mechanisms: by interaction with the gp55 glycoprotein of spleen focus-forming virus (13) and by a point mutation, R129C, in the extracellular domain (14). The presence of a cysteine residue at position 129, and not the loss of an arginine, appears to be required for constitutive activity since substitution of arginine at position 129 with serine, proline, or glutamic acid does not alter the ability of the EPO-R to confer EPO-responsive growth in BA/F3 cells. This requirement for a cysteine residue led to the hypothesis that the constitutive receptor may form intramolecular or intermolecular disulfide bonds, which alter the conformation of the receptor and render it constitutively active.

By analogy with the intramolecular disulfide bonding pattern of the growth hormone receptor (24), it could be predicted that the first and second cysteine residues (C27 and C37) of the EPO-R form a disulfide bond as do the third and fourth (C65 and C81), leaving C179 unpaired. Since the C179S mutant is functionally wild-type, C179 is not essential for normal receptor function, including ligand binding. The phenotype of mutant R129C/C179S demonstrates that C179

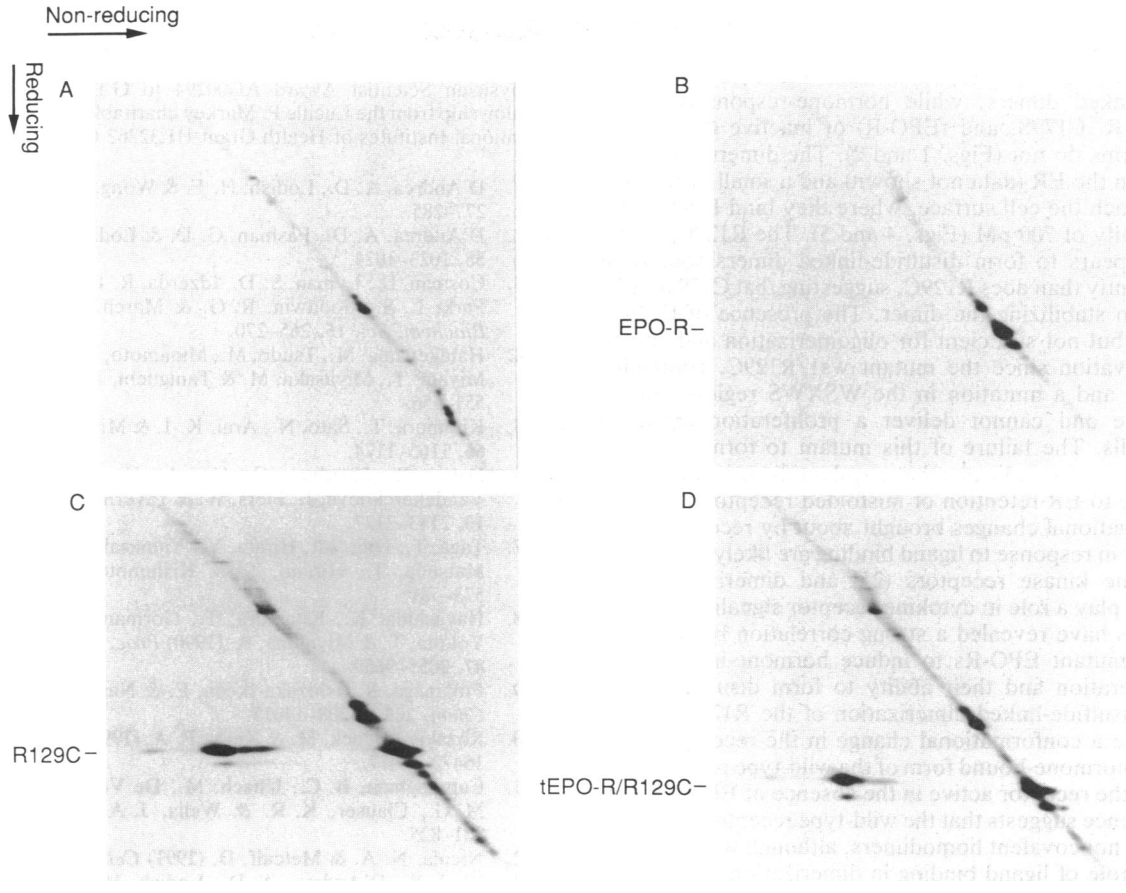

FIG. 3. R129C and tEPO-R/R129C form disulfide-linked homodimers. Parental BA/F3 cells (*A*) or cells expressing the wild-type EPO-R (*B*), R129C (*C*), or tEPO-R/R129C (*D*) were pulse labeled with [^{35}S]methionine/cysteine for 10 min at 37°C and then chased for 2 h at 18°C. The EPO-Rs were immunoprecipitated and separated under nonreducing conditions in the first dimension, followed by reduction and electrophoresis in the second dimension. The positions of monomeric EPO-Rs are indicated.

is also not required for constitutive activity of the receptor; therefore, in the R129C mutant, C179 is unlikely to pair with C129 (Fig. 1). Since the constitutively active R129C receptor binds EPO with an affinity similar to that of the wild-type receptor (Fig. 5), it is unlikely that intramolecular disulfide bond rearrangements have occurred. We conclude that R129C and R129C/C179S form disulfide-linked homodimers through C129.

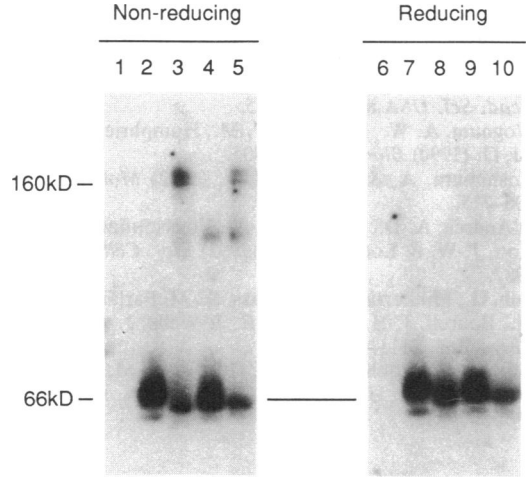

FIG. 4. Disulfide-linked dimers of the constitutive EPO-R are found on the cell surface. Parental BA/F3 cells (lanes 1 and 6) or cells expressing the wild-type EPO-R (lanes 2 and 7), R129C (lanes 3 and 8), C179S (lanes 4 and 9), or R129C/C179S (lanes 5 and 10) were incubated with 10 nM bEPO for 4–6 h at 4°C. The cells were washed and then lysed in buffer containing 0.5% Nonidet P-40 and 200 mM iodoacetamide. Surface receptors that had bound bEPO were isolated on streptavidin-agarose beads, separated by nonreducing and reducing gel electrophoresis, and assayed by immunoblotting. The positions of cell-surface monomeric EPO-Rs (66 kDa) and surface disulfide-linked dimers (160 kDa) are indicated.

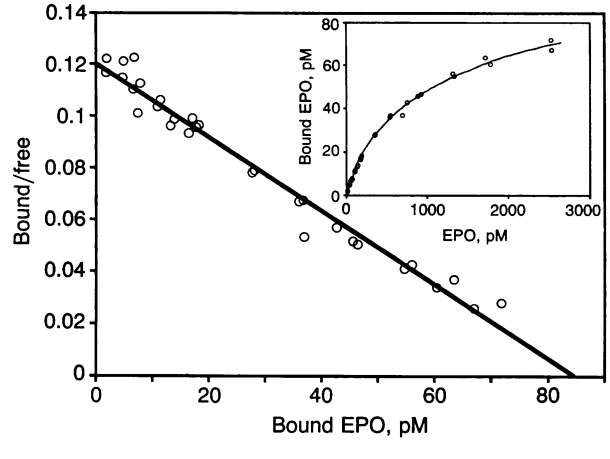

FIG. 5. EPO binding to BA/F3 cells expressing R129C. Approximately 5×10^6 cells expressing R129C were incubated with various concentrations of ^{125}I-labeled EPO, in the presence and absence of 60 nM unlabeled EPO, in a vol of 100 μl for 14 h at 4°C. Free ^{125}I-labeled EPO was separated from the bound hormone, and specific binding was determined as described (20). A saturation curve (*Inset*) and a Scatchard plot of the data are shown.

The formation of disulfide-linked dimers is correlated with constitutive activity. All constitutively active mutants of the EPO-R (R129C, tEPO-R/R129C, and R129C/C179S) form disulfide-linked dimers, while hormone-responsive (wild-type EPO-R, C179S, and tEPO-R) or inactive (ws1, ws1/R129C) forms do not (Figs. 1 and 2). The dimers appear to assemble in the ER (data not shown) and a small proportion of them reach the cell surface, where they bind EPO with a single affinity of 700 pM (Figs. 4 and 5). The R129C/C179S mutant appears to form disulfide-linked dimers somewhat less efficiently than does R129C, suggesting that C179 may be involved in stabilizing the dimer. The presence of C129 is necessary but not sufficient for oligomerization and constitutive activation since the mutant ws1/R129C, containing both C129 and a mutation in the WSXWS region, fails to oligomerize and cannot deliver a proliferation signal in BA/F3 cells. The failure of this mutant to form disulfide-linked dimers also indicates that covalent dimerization is not simply due to ER retention of misfolded receptors.

Conformational changes brought about by receptor oligomerization in response to ligand binding are likely to activate the tyrosine kinase receptors (25) and dimerization also appears to play a role in cytokine receptor signaling (11, 12). Our results have revealed a strong correlation between the ability of mutant EPO-Rs to induce hormone-independent cell proliferation and their ability to form disulfide-linked dimers. Disulfide-linked dimerization of the R129C mutant may induce a conformational change in the receptor, mimicking the hormone-bound form of the wild-type receptor and rendering the receptor active in the absence of EPO. Preliminary evidence suggests that the wild-type receptor is capable of forming noncovalent homodimers, although we do not yet know the role of ligand binding in dimerization.

C129 is likely to be present at the dimer interface of the disulfide-linked R129C receptors and by extrapolation R129 or neighboring residues may play a role in the noncovalent dimerization of the wild-type receptor. Dimerization of the EPO-R may be analogous to the growth hormone receptor, where it has been shown that two receptor subunits bind to different sites on a single growth hormone molecule, and the receptor dimers are stabilized by growth hormone (11). Additional experiments could be directed toward elucidating the structural features involved in EPO-R dimerization and understanding the role of dimerization in receptor activation.

Note Added in Proof. The crystal structure of the growth hormone receptor–growth hormone complex has recently been determined (26). Sequence comparison between the EPO-R and the growth hormone receptor has revealed that residue 129 of the EPO-R would fall in the region corresponding to the growth hormone receptor dimer interface. These observations support the hypothesis that EPO may signal through formation of a noncovalent homodimer of the EPO-R.

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