The pharmacology of cell differentiation
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The erythropoietin receptor: Structure, activation, and tumorigenesis

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INTRODUCTION

Blood cells continuously originate from low numbers of self-renewing pluripotent stem cells that generate progenitor cells committed to one or a few hematopoietic lineages. Control of these differentiation pathways are determined, in part, through specific growth factors binding to their cognate receptor(s) (1). Abrogation or alteration of this control network may lead to the development of leukemia.

Erythropoietin (EPO) is a serum glycoprotein hormone required for the survival, proliferation, and differentiation of committed erythroid progenitor cells (2). We isolated by expression cloning the murine erythropoietin receptor (EPO-R) cDNA (3); it has sequence homology with other cytokine receptors (4). 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 (5). 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 signalling pathways they initiate following 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 (6, 7, 8, 9, 10), while the ligand binding subunits of the granulocyte colony stimulating factor receptor, prolactin receptor, and growth hormone receptor form homodimers (11, 12, 13). Dimerization has been postulated to yield high affinity receptors and also to provide the first step in the signal transduction pathway (12, 14). Importantly, the crystal structure of the growth hormone receptor-growth hormone complex has recently been determined (15). The

exoplasmic domain contains two sub-domains, each of which is built of seven b strands. In dimerization of the growth hormone receptor, two receptor subunits bind to different sites on a single growth hormone molecule. The receptor dimers are stabilized by growth hormone as well as by interactions between the membrane-proximal sub-domains of the exoplasmic segments of the two subunits (15).

Expression of the cloned EPO-R cDNA in the IL-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 (16). The recent demonstration that the mutation Arg129 to Cys (R129C) results in a constitutively active form of the EPO-R, often called cEPO-R, is provocative in that it implicates the formation of aberrant inter- or intramolecular disulfide bonds in the process of receptor activation (17). We investigated the role of the novel cysteine residue in the constitutively active receptor and the possibility that this receptor may have an altered disulfide bonding pattern, using both biochemical and mutagenesis approaches. A cysteine at residue 129 is required for EPO-independent signalling. 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 (18).

Friend virus induces an acute erythroleukemia in adult mice (19). A replication-defective spleen focus-forming virus (SFFV) and a replication-competent Friend murine leukemia virus (F-MuLV) comprise the Friend virus complex. The SFFV env gene encodes a recombinant/ deletion membrane glycoprotein, gp55, that is directly involved in leukemogenesis. The gp55 protein binds to and activates the erythropoietin receptor (EPO-R), resulting in factor-independent cellular proliferation (16, 20). Infecting mice with a SFFV encoding erythropoietin (EPO), rather than gp55, also results in erythrocytosis and splenomegaly similar to early Friend disease (21). Activation of the EPO-R by gp55 may contribute to the early "preleukemic" polyclonal erythroblastosis (22) observed in Friend disease. Consistent with the multistep nature of carcinogenesis, it is apparent that multiple oncogenic events, acting at differing cellular sites, are neccessary to achieve the full leukemic phenotype (23, 24).

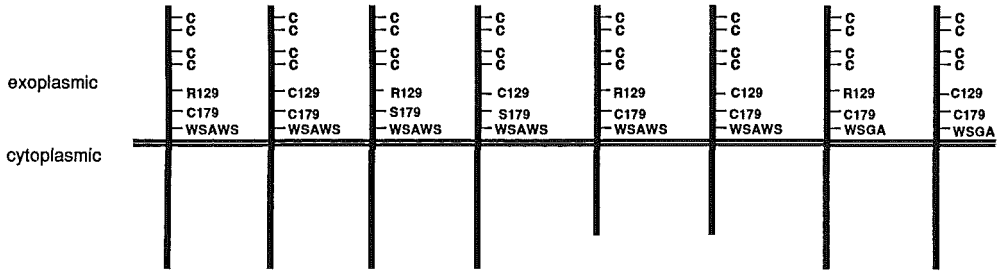
To determine whether the activated erythropoietin receptor could contribute to the development of leukemia we generated a SFFV in which gp55 was replaced with the activated erythropoietin receptor, R129C. This virus, when injected into adult mice, results in polycythemia and splenomegaly. From infected mice we have isolated clonal hematopoietic growth factor-independent cell lines expressing EPO-R-R129C, that are arrested at the proerythroblast stage of erythroid differentiation. These cells when injected back into mice result in rapid development of erythroleukemia (25). This is the first demonstration of a truly oncogenic point mutation in any member of the cytokine receptor superfamily. Consistent with the multistep nature of carcinogenesis, it is apparent that multiple oncogenic events, acting at differing cellular sites, are neccessary to achieve the full leukemic phenotype (23, 24, 26). Importantly, EPO-R R129C does not transform fibroblasts (25); this provides an explanation why oncogenic mutations in other members of this receptor superfamily have not so far been described.

RESULTS

A constitutively active and dimeric erythropoietin receptor

The substitution Arg129 to Cys introduces a sixth cysteine residue into the EPO-R exoplasmic domain and confers constitutive (hormone-independent) activity (Fig. 1, mutant R129C; also called cEPO-R, (17)). Mutation of residue 129 to Ser, Glu or Pro, 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 Cys179. In order to test this hypothesis, Cys179 was mutated to a serine residue either in the wild-type receptor (C179S) or 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 Cys179 is not involved in EPO-induced activation of the wild-type receptor or in constitutive activation of the R129C mutant.

To determine if 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 non-reducing SDS-PAGE. Both the newly synthesized wild-type



Growth in:	EPO-R	R129C	C179S	R129C/C179S	tEPO-R	tEPO-R/R129C	ws1	ws1/R129C
EPO	+	+	+	+	+	+	-	-
No Factor	-	+	-	+	-	+	-	-
Oligomerization	-	+	-	+	-	+	-	-

Figure 1. Schematic diagram of mutant EPO-Rs. Mutants of the EPO-R cDNA were generated or isolated as described in Materials and Methods. The receptors were assayed for their ability to confer EPO-dependent or factor-independent growth in Ba/F3 cells as described (16, 17). Assays for oligomerization were performed as indicated in the legend to Figure 2 and in (18). Residues 129 and 179 are indicated; the four conserved exoplasmic cysteine residues (Cys27, Cys37, Cys65 and Cys81) and the conserved WSAWS sequence (residues 207-211) are not numbered.

EPO-R and R129C migrate with an apparent molecular weight of 64,000 under reducing conditions (27). Under non-reducing conditions the newly synthesized wild-type EPO-R migrates with an apparent molecular weight of 64,000, while the constitutive receptor migrates as a monomer of M_r~64,000 as well as an oligomer of M_r~160,000. The constitutive, truncated EPO-R (tEPO-R/R129C) also forms disulfide-linked oligomers which migrate faster than the R129C oligomers, as expected for an oligomeric species composed of truncated receptor molecules (18).

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 which were generated by reduction of the disulfide-linked oligomers were monomers of R129C (Fig. 2C) and the faster-migrating monomers of tEPO-R/R129C (Fig. 2D). This demonstrates that the disulfide-linked species are EPO-R homo-oligomers, and eliminates the possibility that the constitutive EPO-R forms a heteroligomer with another polypeptide of similar size. The ability of R129C/C179S, but not the wild-type receptor or C179S, to form disulfide-linked oligomers (data not shown) suggests that only Cys129 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. 2B).

We assayed several other 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 (Fig. 1). Inactive receptor mutants include ws1 and ws1/R129C, which lack three residues, Ala-Trp-Ser, from the conserved WSXWS region and which have a Gly-Ala substitution in their place (28). 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 IL-3 (Fig. 1).

To determine if the disulfide-linked homodimers are present on the cell surface we used biotinylated EPO (bEPO) to isolate cell-surface receptors (29). Both R129C monomers and disulfide-linked dimers were found on the plasma membrane (Fig. 3A, lane 3). Similarly, cells synthesizing R129C/C179S (Fig. 3A, lane 5) and tEPO-R/R129C (data not shown) expressed both monomers and dimers on the surface. Following reduction, oligomers of the constitutive

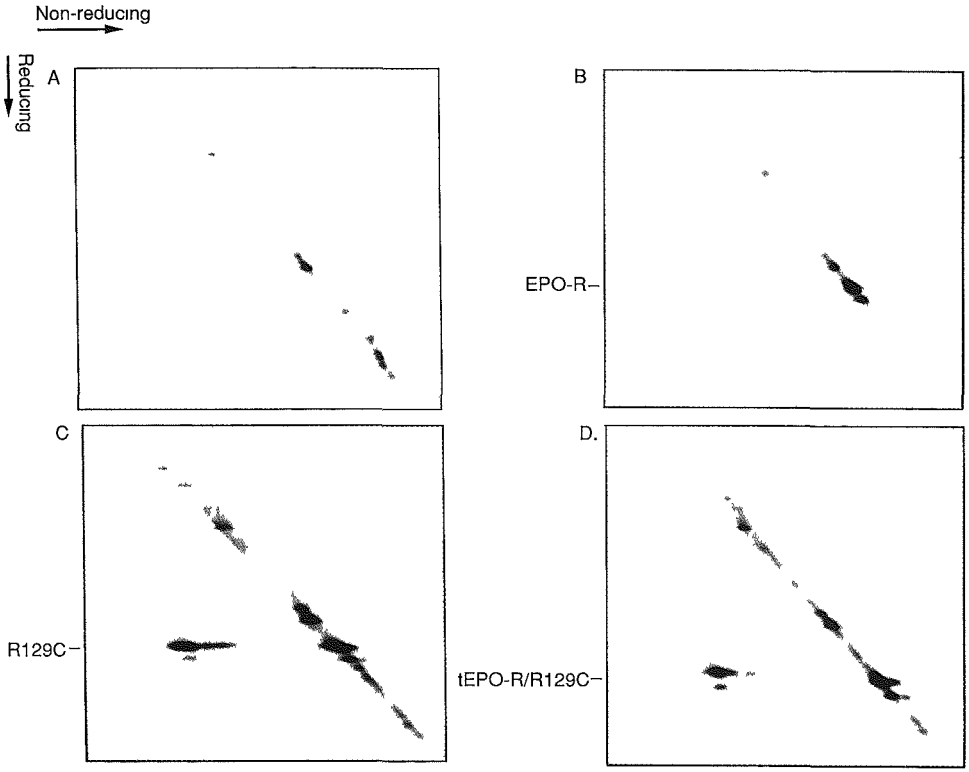


Figure 2. R129C and tEPO-R/R129C form disulfide-linked homodimers. Parental Ba/F3 cells (A) or cells expressing the wild-type EPO-R (B), R129C (C), or tEPO-R/R129C (D) were pulse-labeled with ^{35}S-methionine/cysteine for 10 min at 37°C and then chased for 2h at 18°C. The EPO-Rs were immunoprecipitated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

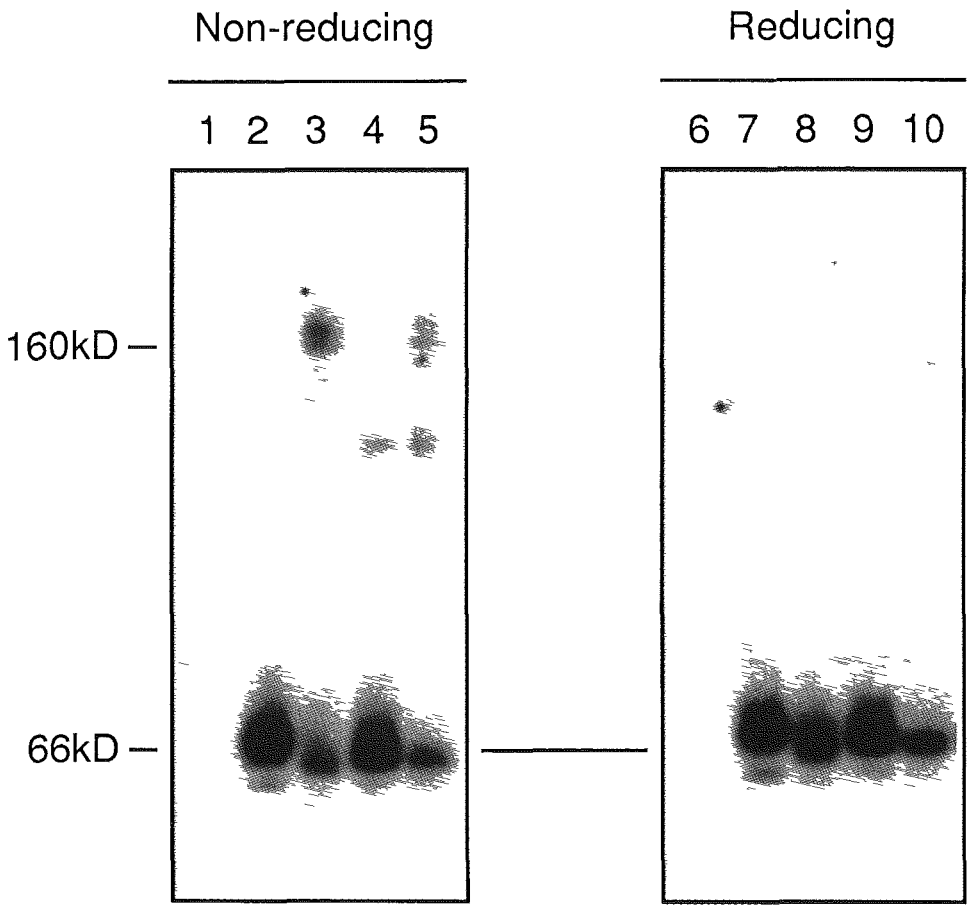


Figure 3. Disulfide-linked dimers of the constitutive EPO-R are found on the cell surface. Parental Ba/F3 cells (lanes 1, 6) or cells expressing the wild-type EPO-R (lanes 2, 7), R129C (lanes 3, 8), C179S (lanes 4, 9), or R129C/C179S (lanes 5, 10) were incubated with 10nM bEPO for 4-6h at 4°C. The cells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

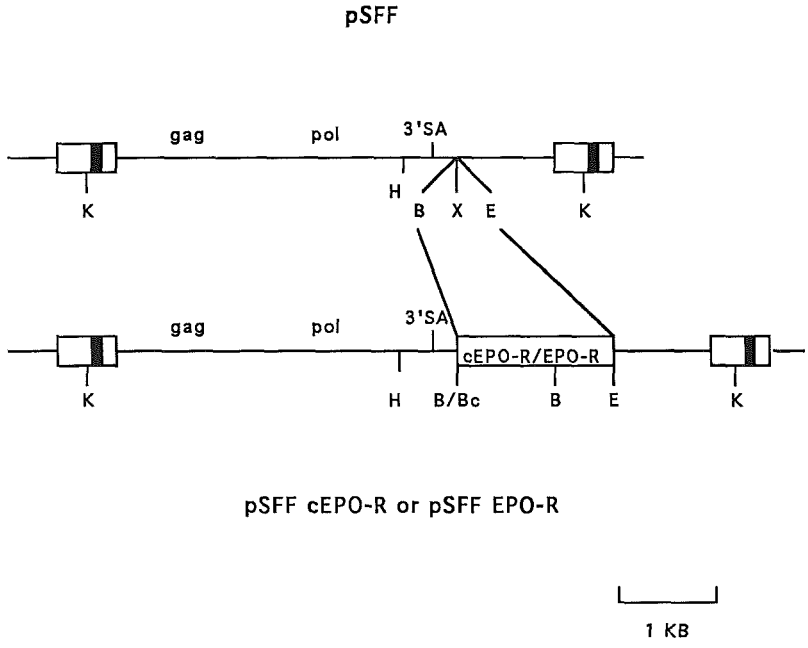


Figure 4. Plasmids used for the generation of viruses. The Kpn1-EcoR1 fragment from pXM cEPO-R or pXM EPO-R, encompassing the coding region of EPO-R R129C or EPO-R cDNAs, was subcloned into the BamH1-EcoR1 site of the truncated env gene of the plasmid pSFF. Unique restriction sites are shown: B, BamH1; Bc, Bcl1; E, EcoR1; H, HindIII; K, Kpn1; X, Xho1. See (25).

receptor were not detectable and only monomers were seen (Fig 3B, 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 (Fig. 3, lanes 2 and 4, data not shown).

Infection of mice with a virus expressing EPO-R R129C (cEPO-R) results in erythrocytosis and splenomegaly.

To test whether EPO-R R129C (cEPO-R) is oncogenic in vivo and could contribute to the development of leukemia we engineered a SFFV in which the pathogenic env gene (gp55) was replaced with the cDNA encoding the constitutively activated erythropoietin receptor (Figure 4). Control experiments showed that the virus, called SFFV cEPO-R, was of substantial titer and biologically functional: infection of IL-3-dependent, pro-B BaF3 cells (30) or

EPO-dependent erythroleukemic HCD57 cells (20) efficiently conferred factor-independent growth (data not shown). This showed, consistent with previous experiments (17), that EPO-R R129C (cEPO-R) can induce factor-independent proliferation of certain non-erythroid as well as erythroid cells.

Initial animal experiments utilized a viral complex generated by rescuing the defective SFFV cEPO-R virus from clones of NIH 3T3 cells by superinfecting these cells with a replication- competent Rauscher MuLV (R-MuLV) helper virus. Five weeks after injecting the resulting viral supernate into four adult Balb/c mice, one mouse (A1) developed erythrocytosis (hematocrit 65, normal 49) and splenomegaly (1.2 g, normal 0.1 g). Blood smears revealed extensive reticulocytosis, evidence of immature erythroid precursors in the blood, and occasional blast-like cells. The splenic architecture was entirely distorted with regression of the white pulp and infiltration with large basophilic blast-like cells. Within the spleen, cells in various stages of erythroid differentiation were evident as was an increase in the number of megakaryocytes (data not shown).

Southern blot analysis of splenic DNA digested with Kpn1, which cuts only in the LTRs of the SFFV cEPO-R provirus, revealed proviral integration (data not shown). Importantly, gp55 was not expressed; this excludes the likelihood of a recombinational event generating wild type SFFV that could cause the observed disease (25).

In subsequent experiments we infected mice with viruses from culture supernatents of SFFV cEPO-R-producing cells mixed with culture supernatents from R-MuLV-producing cells in a 7:3 ratio, respectively. Injection of adult NIH/Swiss mice with this viral complex resulted in Friend-like disease in all mice (data not shown). The latency of disease was again 5 weeks (range 4-6 weeks). Histologic examinations of blood and spleens were similar to those seen with mouse A1. Five mice infected with R-MuLV alone and five mice infected with a SFFV expressing wild type EPO-R (SFFV EPO-R) did not develop polycythemia or splenomegaly. We conclude that the polycythemia and splenomegaly observed in infected mice was directly related to the SFFV virus expressing cEPO-R (EPO-R R129C).

Characterization of leukemic, growth factor-independent cell lines established from diseased spleens.

Growth factor-independent cell lines were derived from the spleens of

Table 1. Characterization of cell lines established from the spleen of a mouse infected with SFFV-cEPO-R

Cytokine-independent proliferation	+
Leukemogenic	+
SFFV cEPO-R proviral integration	+
cEPO-R proviral mRNA	+
EPO-R R129C protein	+
gp55 protein	-
spi-1 activation	-
p53 gene rearrangement and inactivation	+
GATA-1 mRNA	+
Globin mRNA	+
Band 3 mRNA	-
B220 protein	-
Thy-1 protein	-
MAC-1 protein	-

mice A1 and D1. Most analyses were carried out on 4 of 13 cell lines derived from the spleen of mouse A1. Histologically the growth factor-independent cell lines appear similar to the basophilic blast-like cells seen in the diseased spleens (data not shown).

Table 1 summarizes the characteristics of growth factor-independent cell lines isolated from the spleen of a mouse infected with SFFV cEPO-R. All cell lines examined express GATA-1, a DNA-binding protein present at all stages of erythroid development yet also present in megakaryocytes and mast cells (31). These cell lines express globin, but do not express the erythroid anion transporter Band 3, or markers of B cells, T cells, or macrophages (25). Thus the cell lines isolated appear to be arrested at the proerythroblast stage of erythroid differentiation.

Demonstration that factor-independent cell lines are leukemogenic

Substantial evidence indicates that two of the cell lines tested (A1.4 and A1.5) are directly leukemogenic. To determine whether the growth factor-independent, clonal, proerythroblast cell lines isolated were fully transformed and leukemogenic, syngeneic mice were injected intravenously with 1-5 x 10^6 cells of clone A1.4 or A1.5. After 2 weeks mice receiving intravenous injections of cells became severely anemic (hematocrit 20-24, normal 49) without evidence of reticulocytosis. The total white blood cell and platelet counts were reduced. They also had significant splenomegaly (0.4-0.6 g, normal 0.1 g). Histologic sections of the spleen and cytospin preparations from the bone marrow show total replacement of these organs with basophilic, leukemic blast-like cells with a high mitotic index (data not shown). Within the bone marrow and spleen there was no evidence of erythroid differentiation past the proerythroblast stage. Grossly, the bone marrow appeared white. Examination of cytospin preparations from the bone marrow of these mice revealed complete replacement of the marrow cavity with erythroblastic appearing cells similar to the injected cells(data not shown). Immunoblot analysis of proteins extracted from the leukemic spleens and bone marrow detected EPO-R R129C expression without evidence for gp55 expression (25).

Importantly, mice injected intravenously with A1.4 or A1.5 cells did not, at any time, develop erythrocytosis which would be indicative of infection with a SFFV cEPO-R virus secreted by the injected clones. Rather they became severely anemic, and their bone marrow compartments were completely replaced with cells morphologically and biochemically similar to A1.4 or A1.5. Other evidence (25) supports the clonal nature of the erythroleukemia induced by the injected cell lines, and demonstrates that the erythropoietin receptor expressed in diseased spleens and growth factor-independent cell lines retains the activating mutation.

DISCUSSION

Dimerization of the EPO-R

The EPO-R can be activated by two different, hormone-independent mechanisms; by interaction with the gp55 glycoprotein of spleen focus-forming virus (16) and by a point mutation, Arg129 to Cys, in the extracellular domain (17). 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

Arg129 with Ser, Pro or Glu 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 novel 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 (15) it could be predicted that the first and second cysteine residues (Cys27 and Cys37) of the EPO-R form a disulfide bond as do the third and fourth (Cys65 and Cys81), leaving Cys179 unpaired. Since the C179S mutant is functionally wild-type, Cys179 is not essential for normal receptor function, including ligand binding. The phenotype of mutant R129C/C179S demonstrates that Cys179 is also not required for constitutive activity of the receptor, therefore in the R129C mutant Cys179 is unlikely to pair with Cys129 (Fig. 1). Since the constitutively active R129C receptor (cEPO-R) binds EPO with an affinity similar to that of the wild-type receptor (18) it is unlikely that intramolecular disulfide bond rearrangements have occurred. We conclude that EPO-R R129C and EPO-R R129C/C179S form disulfide-linked homodimers through Cys129.

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 forms (ws1, ws1/R129C) do not (Figure 1). The dimers appear to assemble in the ER (data not shown) and a small proportion of them reach the cell surface (Fig. 3), where they bind EPO with a single affinity of 700 pM (18). The presence of Cys129 is necessary but not sufficient for oligomerization and constitutive activation since the mutant ws1/R129C, containing both Cys129 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.

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 non-covalent homodimers, though we do not yet know the role of ligand binding in dimerization.

The crystal structure of the growth hormone receptor-growth hormone complex is known (15). Sequence comparison between the EPO-R and the growth hormone receptor reveals that residue 129 of the EPO-R would fall in the membrane-proximal subdomain of the growth hormone receptor, at the dimer interface. Thus, Cys129 is likely to be present at the dimer interface of the disulfide-linked R129C receptors and, by extrapolation, Arg129 or neighboring residues may play a role in the non-covalent dimerization of the wild-type receptor. Indeed, we have recently generated mutants in which the amino acids at positions 132 or 133 of the EPO-R are changed to cysteine. Both of these mutants allows factor-independent proliferation of normally IL-3-dependent cells, indicating that residues 132 and 133 are, as predicted by the structure of the growth hormone receptor, also at the dimer interface (data not shown).

Induction of erythroleukemia by the EPO-R R129C.

The mutant cEPO-R (EPO-R R129C) transduces a proliferative signal in the absence of any added growth factor; expression of EPO-R R129C in IL-3-dependent BaF3 cells (17) or EPO-dependent HCD57 cells results in deregulation of cellular proliferation manifest as growth factor-independence. To test whether this activating mutation in the erythropoietin receptor can contribute to the development of leukemia we generated a SFFV in which the pathogenic env gene, gp55, was replaced with the cDNA encoding EPO-R R129C. Mice injected with this virus (SFFV cEPO-R) developed polycythemia and splenomegaly. From the spleen of infected mice we isolated factor-independent cells arrested at the proerythroblast stage of development which produced leukemia when injected into syngeneic mice.

Early in the course of Friend virus infection there is a polyclonal erythroblastosis, without any increase in the self-renewal capacity or abrogation of differentiation potential of the cell (22). After 4-6 weeks clonal, growth factor-independent, leukemogenic cells evolve (32, 33), which are arrested at the proerythroblast stage of differentiation. Activation of the EPO-R by gp55 is thought to be responsible for the early erythroblastosis, which could allow for subsequent genetic events that would result in the promotion of the leukemic clone, altered in its differentiation program. The role of gp55 in the later stages of Friend virus-induced erythroleukemia is unclear. We have shown that the structurally activated EPO-R R129C can supplant gp55 as an

oncogenic agent in Friend virus-induced erythroleukemia. Presumably EPO-R R129C mimics gp55 in promoting uncontrolled erythroblastosis allowing for further genetic events to occur that would promote the evolution of the full leukemic phenotype. As with gp55 the role, if any, of EPO-R R129C in the later stages of the disease are unclear.

Later events thought to contribute to the evolution of immortal, leukemic transformation in SFFV-induced erythroleukemia include insertional activation of the putative oncogene spi-1 (34), and/or the inactivation of the suppressor oncogene p53 (24, 35). Since the leukemic cell lines described in this report were derived from infection of mice with a modified SFFV, SFFV cEPO-R, we expected to find activation of spi-1 gene expression. However, individual leukemic clones derived from this pool did not express spi-1, nor was there evidence for proviral integration in the spi-1 genomic locus. Thus, in contrast to erythroleukemia induced by Friend virus, activation of spi-1 is unnecessary to achieve the full leukemic phenotype.

Both alleles of the p53 gene of the leukemic clone A1.5 were rearranged, and this resulted in inactivation of p53 gene expression (25). Presumably the p53 gene rearrangement in cell line A1.5 occured in vivo and confered a selective growth advantage, a situation similar to leukemic clones derived from infections with Friend virus. Whether or not p53 inactivation alone is sufficient to generate the fully transformed leukemic phenotype in these erythroleukemic cells remains to be determined.

Following infection of mice with SFFV cEPO-R there were elevations only in the number of circulating erythroid cells (erythrocytosis) and only erythroleukemic cell lines were isolated from the spleen. One would expect that factor-independent proliferation of cells expressing EPO-R R129C, as a result of SFFV cEPO-R infection, would be limited to those that can transduce a proliferative signal from the EPO-R R129C. Yet the disease caused by SFFV cEPO-R has only erythroid manifestations. The EPO-R R129C is capable of confering factor-independent growth upon a line of IL-3-dependent pre-B cells, BaF3 (16, 17), an IL-2-dependent cytotoxic T cell line, CTLL-2 (data not shown), and an EPO-dependent erythroid cell line, HCD57. Thus our results suggest that either the SFFV LTR restricts expression of EPO-R R129C to red cell precursors, or in vivo EPO-R R129C is only capable of transducing a signal in erythroid cells.

That EPO-R R129C did not transform fibroblasts results from the inability of EPO-R to transduce a proliferative signal in these cells. Presumably fibroblasts lack some molecule(s), inherent to erythroid cells, necessary for signal transduction by members of the cytokine receptor superfamily. This may explain why, to date, oncogenic activations in members of the cytokine receptor superfamily have not been observed using fibroblasts in transformation assays.

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Discussion - THE ERYTHROPOIETIN RECEPTOR: STRUCTURE, ACTIVATION, AND TUMORIGENESIS

M. Oren

Would your activated receptor retrovirus render cells like BF3 and 32D factor-independent for growth, and if the answer is yes, and since that receptor does not seem to be implicated in myeloid cell growth, could you make any kind of guess as to what the difference between those and normal myeloid cells is?

H.F. Lodish

I think that the best guess, at this point, is that one or more of the downstream signalling molecules are lineage specific, be they be protein tyrosine kinases or not. A point that I should have made, but didn't, is that the EPO receptor does not signal in fibroblasts. That is, if you put the constitutively active receptor in fibroblasts it has no effect on their growth or proliferation. Interestingly, the EPO receptor itself is not tyrosine phosphorylated in fibroblasts when EPO is added, so at least part of that signalling pathway is missing in fibroblasts. This is, I think, one of the reasons why if there are oncogenic mutations in other members of the cytokine receptor super-family, they have not been detected, is that everyone uses fibroblasts as an assay. I think it also argues that at least the tyrosine kinases are lineage specific, at least the kinase that phosphorylates the EPO receptor. But it's hard to be more specific until we know what the downstream signalling molecules are.

J. Massagué

Your evidence regarding the dimerization shows that one receptor monomer is disulfide bonded to something else.

H.F. Lodish

Yes. We have made a double mutant receptor that is both constitutive and has the C terminal 40 aa truncation. When cells that express that receptor are labelled and then analyzed by 2 D gels, we see a single off diagonal spot that is smaller than the

full-length EPO-R. So unless the second protein is not labelled with methionine or cysteine, I think one has to argue that it is a homodimer.

J. Massagué

There is still the possibility the receptor forms a dimer with something else, and this would also be very interesting. Would you be able to form the dimer with an antibody, for example, test divalent antibody versus a Fab fragment, and check whether dimerization by antibody activates the receptor?

H.F. Lodish

Yes, these are the sorts of experiments that have been very difficult to do because the number of cell surface receptors in a cell is only about a thousand. Despite a lot of efforts we can't do the direct experiments; we cannot label the surface of a cell with radioiodine and "see" the receptor. We have tried, but we can't do the experiment that you would like. We have looked at antibodies to the receptor. We have made antibodies to the N-terminal peptide. These do not seem to stimulate proliferation, but that is a negative result. It would be nice if they did. Could the EPO-R perhaps be dimerized to some other molecule that is too big to enter the gel? I think it's unlikely, but strictly speaking we can't disprove it.

J. Massagué

Do you have any evidence whether when the receptor dimerizes it recruits the signalling subunit?

H.F. Lodish

Again, we cannot do the experiment. The only way we can get at the cell surface receptors is by affinity purifying whatever binds biotinylated EPO, and in that complex we can show both the tyrosine kinase and the two molecules that were phosphorylated - one was the receptor and the other was this 130 kD protein - so we presume that the 130 kD is involved in the signalling pathway. But the way that we did the experiments, using biotinylated EPO, we can't ask the question of who's together

beforehand. However - parenthetically - this is just not my problem. The people who work on the IL-2 receptor, where you have both alpha and beta subunits, the beta being in the cytokine receptor family, and now apparently a gamma subunit, have the same problem: they can't really tell whether the subunits are bound, are together in the absence of ligand. For that matter, with the TGF-β receptor, one also has trouble doing that, so I think that a lot of these systems share the same kind of problem in asking whether the receptor is brought together by the ligand or whether the complex pre exists. We really don't really know that.

R. Benezra

I was just wondering if it is the availability of EPO during the synthesis of the receptor that normally prevents erythroleukemia. If not, how does the system shut down normally?

H.F. Lodish

EPO is made normally throughout the life span, but interestingly one group has made transgenic mice that overexpress EPO. In this case one gets polycythemia, but one doesn't get tumours. So the idea is EPO itself stimulates proliferation, but there's probably some desensitisation mechanism that is activated and that prevents this continuing high level signalling being sent. On the other hand, when you are expressing an activated receptor you by-pass all that and the cells just keep cycling. That is probably why you get tumours with the activated receptor and not with the EPO.