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Activation and Inhibition of Erythropoietin Receptor Function: Role of Receptor Dimerization

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Members of the cytokine receptor superfamily have structurally similar extracellular ligand-binding domains yet diverse cytoplasmic regions lacking any obvious catalytic domains. Many of these receptors form ligand-induced oligomers which are likely to participate in transmembrane signaling. A constitutively active (factor-independent) mutant of the erythropoietin receptor (EPO-R), R129C in the exoplasmic domain, forms disulfide-linked homodimers, suggesting that the wild-type EPO-R is activated by ligand-induced homodimerization. Here, we have taken two approaches to probe the role EPO-R dimerization plays in signal transduction. First, on the basis of the crystal structure of the ligand-bound, homodimeric growth hormone receptor (GH-R) and sequence alignment between the GH-R and EPO-R, we identified residues of the EPO-R which may be involved in intersubunit contacts in an EPO-R homodimer. Residue 129 of the EPO-R corresponds to a residue localized to the GH-R dimer interface region. Alanine or cysteine substitutions were introduced at four other residues of the EPO-R predicted to be in the dimer interface region. Substitution of residue E-132 or E-133 with cysteine renders the EPO-R constitutively active. Like the arginine-to-cysteine mutation at position 129 in the exoplasmic domain (R129C), E132C and E133C form disulfide-linked homodimers, suggesting that constitutive activity is due to covalent dimerization. In the second approach, we have coexpressed the wild-type EPO-R with inactive mutants of the receptor missing all or part of the cytosolic domain. These truncated receptors have a dominant inhibitory effect on the proliferative action of the wild-type receptor. Taken together, these results strengthen the hypothesis that an initial step in EPO- and EPO-R-mediated signal transduction is ligand-induced receptor dimerization.

Ligand-induced oligomerization of growth factor receptors plays a crucial role in signal transduction by bringing receptor cytoplasmic domains into proximity such that they are rendered competent to bind and activate downstream signaling molecules. This mechanism of transmembrane signaling has been found for members of the tyrosine kinase receptor family, such as c-Kit and the epidermal growth factor receptor (EGF-R); ligand-induced oligomerization activates their cytoplasmic kinase domains, leading to receptor phosphorylation and interaction with intracellular signaling proteins (29, 50). Receptors for the hematopoietic growth factors compose a new family and are defined by regions of similarity in their exoplasmic domains and which lack kinase-related sequences in their cytoplasmic domains (2, 6). Although phosphorylation of cytosolic proteins is a consequence of receptor-ligand binding (24, 27, 35), the precise modes of signal transduction for members of the cytokine receptor family remain to be defined. Oligomerization of cytokine receptors in response to ligand binding is likely to be an essential first step in the signaling pathway (10, 26, 36), generating complexes which bind and activate intracellular, nonreceptor tyrosine kinases (1, 44, 52).

The oligomeric structures of the members of the cytokine receptor family are varied and complex. The interleukin-2 (IL-2) receptor is composed of three membrane-bound polypeptides, α, β, and γ (48); mutation of the latter subunit has recently been implicated as the genetic defect in human X-linked severe combined immunodeficiency (39). The high-affinity forms of the IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor receptors are heterooligomers composed of a ligand-specific α-subunit and a common β-subunit (12, 18, 19, 26, 28, 46, 49). Ligand-induced heterooligomerization of α- and β-subunits is required for proliferation in physiological concentrations of hormone (26, 27). Similarly, the high-affinity receptors for leukemia-inhibitory factor, IL-6, ciliary neutrotrophic factor, and oncostatin M have distinct, as well as common, components which oligomerize in response to ligand binding to initiate the signaling cascade (10, 16, 20, 23, 45).

In contrast, some other members of the cytokine receptor family form homodimers following ligand binding, such as the receptors for growth hormone (GH), prolactin, and granulocyte colony-stimulating factor (8, 13–15, 22, 43). Homodimerization of the erythropoietin receptor (EPO-R) also appears to be critical for signal transduction. A constitutively active (hormone-independent) EPO-R, containing an arginine-to-cysteine mutation at position 129 in the exoplasmic domain (R129C), was isolated following retroviral transduction (55). Since substitution of position 129 with Glu, Pro, or Ser generates receptors that require EPO for activation, the presence of cysteine and not the loss of arginine at position 129 is necessary for hormone-independent proliferation. The R129C mutant forms disulfide-linked homodimers in the absence of EPO; these are found both in internal membranes and on the plasma membrane (51). We hypothesized that the disulfide-linked dimers might mimic the structure of the hormone-bound form of the wild-type EPO-R, which may be activated by ligand-induced dimerization, and thus the covalently dimerized receptors are able to transmit a constitutive proliferative signal (51).

The structure of the GH-GH receptor (GH-R) complex

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provides a paradigm for the structure of other homodimeric cytokine receptors (11). The hormone-receptor complex forms an unusual structure, since two receptor molecules are bound to a monomeric, nonsymmetrical ligand molecule (8, 11). Similar binding determinants in each receptor monomer interact with distinct sites (site I and site II) on GH (11). Receptor dimerization is essential for signal transduction and occurs by sequential binding; one GH-R molecule binds to site I on GH, and then a second binds to site II (8, 14). Residues in the membrane-proximal domains of the two GH-R monomers (termed here the dimer interface) interact with each other and stabilize the ligand-bound dimer (11). Ligand-induced homodimerization stimulates interaction with the tyrosine kinase JAK2, which may initiate a signaling cascade, culminating in cell proliferation (1).

In this study we have utilized two distinct approaches to test the hypothesis that the EPO-R is activated by homodimerization. In the first, we introduced cysteine point mutations into the putative EPO-R dimer interface region, identified by comparison with the GH-R structure, and identified new constitutively active, disulfide-linked homodimeric forms of the EPO-R. The extracellular domains of the cytokine receptors share a similar genomic organization, have approximately 20% amino acid identity, and are thought to form similar tertiary structures (2, 6). The GH-R crystal structure demonstrates that many of the highly conserved residues maintain the three-dimensional structure of the molecule, making it likely that other members of the family adopt a similar conformation (11). Here we show by alignment of the exoplasmic domain sequences of the GH-R and the EPO-R that R-129 of the EPO-R falls in the region corresponding to the dimer interface of the GH-R and that other residues of the EPO-R in proximity to R-129 are also predicted to lie in this region. Mutation of residues flanking R-129 to cysteine generates two novel, hormone-independent forms of the EPO-R. Similar to the R129C receptors, these mutant EPO-Rs form disulfide-linked homodimers.

In the second approach, we generated and assayed mutants of the EPO-R deleted in all or part of their cytoplasmic domains. Unlike the wild-type EPO-R, these mutant receptors cannot stimulate EPO-dependent cell proliferation when expressed in IL-3-dependent hematopoietic cells. When these mutants were coexpressed with the wild-type EPO-R in an EPO-dependent cell line, they inhibited the proliferative activity of the receptor. Coexpression of the truncated receptors did not affect the biosynthesis of the wild-type EPO-R, and cell lines containing both wild-type and truncated receptors exhibited a 2- to 50-fold increase in the number of surface EPO binding sites. These results suggest that the inhibitory activity of the truncated receptors is due to ligand-induced oligomerization between wild-type and truncated receptors at the cell surface.

MATERIALS AND METHODS

Mutagenesis techniques. The dimer interface mutants were constructed by a cassette mutagenesis approach. Briefly, pBluescript, containing the murine EPO-R cDNA (9) cloned into the *Kpn*I site, was partially digested with *Sac*II and completely with *Bsa*AI, and then the DNA was dephosphorylated. The partially complementary oligonucleotides RRAEE 1 and RRAEE 2 were annealed to each other and then ligated to the plasmid DNA. RRAEE 1 is 5′-471-GGGCCTGCTGGC GCCCGCAGCAGCAGCGATATCACAT-507-3′, and RRA EE 2 is 5′-507-ATGTGATATCGCTGCTGCTGCGGGCGC CAGCAGGCCCGC-468-3′, by the numbering system in reference 9. Plasmid minipreparations were screened by restriction enzyme digestions, and a clone which had the oligonucleotides ligated at the desired position (substituting nucleotides 471 to 507 of the EPO-R cDNA) was isolated. This cassette construct has a unique *Nar*I site at position 481 and a unique *Eco*RV site at position 500 of the EPO-R cDNA, and the sequences 5′ to the *Nar*I site and 3′ to the *Eco*RV site encode amino acids corresponding to the wild-type EPO-R. The cDNA insert encoding the cassette version of the EPO-R was then subcloned into expression vector pXM (53) that had been altered to remove its *Eco*RV site. This construct was digested with *Nar*I and *Eco*RV and dephosphorylated, and pairs of oligonucleotides corresponding to each point mutant were then annealed and ligated into the cassette DNA. The oligonucleotide pairs utilized are as follows: R129C 1, 5′-482-CG TGTCGGGCAGAAGAAGG-500-3′, and R129C 2, 5′-500-C CTTCTTCTGCCCGACA-484-3′; R130A 1, 5′-482-CGCGC GCTGCAGAAGAAGG-500-3′, and R130A 2, 5′-500-CCTT CTTCTGCAGCGCG-484-3′; R130C 1, 5′-482-CGCGC TGTGCAGAAGAAGG-500-3′, and R130C 2, 5′-500-CCTTC TTCTGCACAGCG-484-3′; A131C 1, 5′-482-CGCGCCG GTGTGAAGAAGG-500-3′, and A131C 2, 5′-500-CCTTCTT CACACCGGCG-484-3′; E132A 1, 5′-482-CGCGCCGGGC AGCTGAAGG-500-3′, and E132A 2, 5′-500-CCTTCAGCTG CCCGGCG-484-3′; E132C 1, 5′-482-CGCGCCGGGC ATGTGAAGG-500-3′, and E132C 2, 5′-500-CCTTCACATG CCCGGCG-484-3′; E133A 1, 5′-482-CGCGCCGGGCAGAA GCTGG-500-3′, and E133A 2, 5′-500-CCAGCTTCTGCCCG GCG-484-3′; E133C 1, 5′-482-CGCGCCGGGCAGAATGTG G-500-3′, and E133C 2, 5′-500-CCACATTCTGCCCGGC G-484-3′; and AAAAA 1, 5′-482-CGGCAGCCGCTGCGGC AGG-500-3′, and AAAAA 2, 5′-500-CCTGCCGCAGCGGC TGC-484-3′. The nucleotide positions of the EPO-R cDNA sequence to which the oligonucleotides correspond are indicated (see reference 9), and the sequences which encode amino acid changes are underlined. Plasmid minipreparations were prepared, and their sequences were determined to verify that only the desired mutations had been introduced, prior to the mammalian cell transfection experiments.

Construction of the EPO-R cytoplasmic deletion mutants was accomplished by insertion of premature termination codons into the EPO-R coding sequence. Mutants EPO-R/1-306 and EPO-R/1-257 were constructed by *Escherichia coli* exonuclease III digestion of the EPO-R cDNA, as described elsewhere (37a). The precise endpoints of these mutants were determined by dideoxy sequence analysis. EPO-R/1-306 encodes amino acids 1 to 306 of the mature EPO-R plus an additional two amino acids, NK, encoded by the oligonucleotide used to introduce the stop codons. EPO-R/1-257 contains a stop codon immediately following residue 257 of the mature EPO-R. Mutant 1-256/R129C was generated from the EPO-R R129C mutant in the pXM vector by partial digestion with *Bgl*II and insertion of an oligonucleotide encoding a stop codon following residue 256. The truncation mutants were subcloned into the unique *Kpn*I sites in the pXM and pMEX vectors.

Cell culture conditions, transfections, and cell line selection conditions. The untransfected myeloid 32D cell line and the pro-B BA/F3 cell line utilized in this study are strictly IL-3 dependent. Both cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 5% conditioned medium from the WEHI 3B cell line (WEHI CM) as a source of IL-3. Cell lines expressing the wild-type EPO-R cDNA or the mutants R130A, R130C, A131C, E132A, E133A, and AAAAA were maintained in RPMI 1640 medium supplemented with 10% FCS and 1 U of

EPO per ml. Cell lines expressing mutants R129C, E132C, and E133C were maintained in RPMI 1640 medium supplemented only with 10% FCS. The cell lines expressing 1-256/R129C or coexpressing the wild-type EPO-R and EPO-R/1-306 or EPO-R/1-257 were maintained in RPMI 1640 medium supplemented with 10% FCS and 5% WEHI CM.

Transfection of 32D and BA/F3 cells was accomplished by electroporation with a Bio-Rad Gene Pulser. Cells were placed in phosphate-buffered saline at a density of 10^7/0.8 ml, linearized DNAs (20 μg) were added and mixed, and the cells were electroporated at 960 μF and 0.25 V. Cells were placed in RPMI 1640 medium supplemented with 10% FCS and 5% WEHI CM for 48 h at 37°C to recover.

32D cells were cotransfected with cDNAs encoding the dimer interface mutants in the vector pXM (20 μg of test DNA) and pSV2neo (1 μg) and were initially selected either in medium containing IL-3 and G418 (500 μg/ml) or in medium containing 1 U of EPO per ml. All of the cell lines expressing the dimer interface mutants proliferated in response to EPO, and therefore clonal cell lines were isolated by limiting dilution and growth in medium containing 1 U of EPO per ml. BA/F3 cell lines expressing the dimer interface mutants were established by electroporation and selection in medium containing 1 U of EPO per ml.

The 32Dn20 cell line was established by electroporation of 32D cells with the wild-type EPO-R cDNA in pXM and selection in medium containing 1 U of EPO per ml. Clonal lines were isolated by limiting dilution and assayed for EPO-R expression by immunoprecipitation. The 32Dn20 line expresses relatively low levels of the EPO-R (data not shown). This line was then electroporated with either the vector pMEX alone or pMEX containing EPO-R/1-306 or EPO-R/1-257 and transfected cells were selected by growth in medium containing 5% WEHI CM and 500 μg of G418 per ml. Clonal lines were isolated by limiting dilution.

Growth factor-dependent proliferation assays. The ability of the EPO-R dimer interface mutants to confer growth factor-independent proliferation was tested by transferring the cell lines to medium lacking added growth factors. EPO was removed from the medium two different ways, either by 10-fold dilutions of the cells into RPMI 1640 medium containing 10% FCS when they reached a density of ~10^6/ml or by washing the cells three times with RPMI 1640 medium containing 10% FCS and plating directly into RPMI 1640 medium containing 10% FCS and lacking any added EPO.

Cell lines were assayed for EPO-responsive growth by washing the cells three times in RPMI 1640 medium containing 10% FCS to remove EPO or IL-3 and then plating them at 10^4/ml in RPMI 1640 medium containing 10% FCS and the indicated concentrations of EPO, in RPMI 1640 medium containing 10% FCS and 5% WEHI CM, or in RPMI 1640 medium plus 10% FCS. After 3 days in culture, viable cells were counted.

The doubling times of clonal cell lines were determined by passage in RPMI 1640 medium containing 10% FCS and supplemented with 1 to 10 U of EPO per ml (as indicated in the legends), 5% WEHI CM, or no growth factor. Cells were washed three times in RPMI 1640 medium containing 10% FCS prior to plating in the different growth factor conditions and were maintained (by dilution) under a density of 10^6/ml for the duration of the assay. Viable cells were counted each day, for up to 7 days.

Iodination of EPO and equilibrium binding studies. EPO was radioiodinated by the iodine monochloride method (21, 21a) and was routinely labeled to a specific activity of 4×10^6 cpm/pmol. Clonal cell lines expressing the wild-type EPO-R or EPO-R mutants were used for binding studies as described elsewhere (21, 21a).

Immunoprecipitation, SDS-PAGE, and immunoblot analysis. Cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (^{35}S Express; NEN) for the indicated times, and some samples then were incubated in medium containing excess unlabeled amino acids for a 2-h chase period. Immunoprecipitations, with antipeptide antibodies specific for the amino- or carboxy-terminal residues of the murine EPO-R (54), were performed as described previously (51). Immunoprecipitated proteins were separated by one- or two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (31). For two-dimensional SDS-PAGE, proteins were separated under nonreducing conditions in the first dimension and under reducing (5% β-mercaptoethanol) conditions in the second. The gels were subjected to fluorography and were visualized by autoradiography. Immunoblotting was performed as previously described (51).

RESULTS

The intron-exon boundaries of the cytokine receptor genes are highly conserved (for an overview, see reference 2). We aligned the amino acid sequences of the human GH-R and murine EPO-R exoplasmic domains exon by exon. Motifs which are conserved in the cytokine receptor superfamily, such as the four cysteine residues (exons 2 and 3 [not shown]), the proline residue following the GH-R hinge region (exon 4 [Fig. 1A]) and the WSXWS homology sequence (exon 5 [not shown]) were aligned initially, and then identical and homologous amino acids were aligned. Gaps were introduced in each sequence to align identical or conserved residues. Figure 1A shows the portions encoded by exon 4, containing the majority of residues of the GH-R at the dimer interface (11). Residue 129 of the EPO-R, which is mutated to cysteine in the constitutively active EPO-R R129C, aligns with residues of the GH-R which participate in intersubunit interactions. This alignment suggested residues in proximity to R-129 (R-130, A-131, E-132, and E-133) that might also be involved in intersubunit contacts in a ligand-induced homodimer (Fig. 1A).

Generation of mutations in the EPO-R dimer interface. To test this hypothesis, we constructed several mutants in the putative EPO-R dimer interface region (Fig. 1B). Positions 130 through 133 of the EPO-R were altered individually to encode alanine (R130A, E132A, and E133A) or cysteine (R129C, R130C, A131C, E132C, and E133C), and in addition, all five residues (129 to 133) were changed to alanine (mutant AAAAA). As analyzed by transient transfection in COS cells, all mutant receptors are expressed on the cell surface and bind ^{125}I-EPO. In addition, transfected cells expressing the mutant receptors internalized ^{125}I-EPO at rates similar to those of cells expressing the wild-type receptor, as judged by the ratio of internal to cell surface ^{125}I-EPO after a 5-h incubation at 37°C (data not shown).

Proliferative ability of the EPO-R dimer interface mutants. The mutant receptors were assayed for their ability to confer EPO-dependent or hormone-independent proliferation to the IL-3-dependent cell lines 32D or BA/F3. Stable expression of the wild-type EPO-R in these cell lines enables them to proliferate in the presence of EPO as the sole supplemental growth factor (33, 34). Preliminary studies of 32D cells transfected with the EPO-R mutants indicated that all of the mutants enabled cells to proliferate in the presence of 1 U of EPO per ml. Untransfected 32D cells maintained in medium containing either G418 and IL-3 or EPO alone died after 3 to

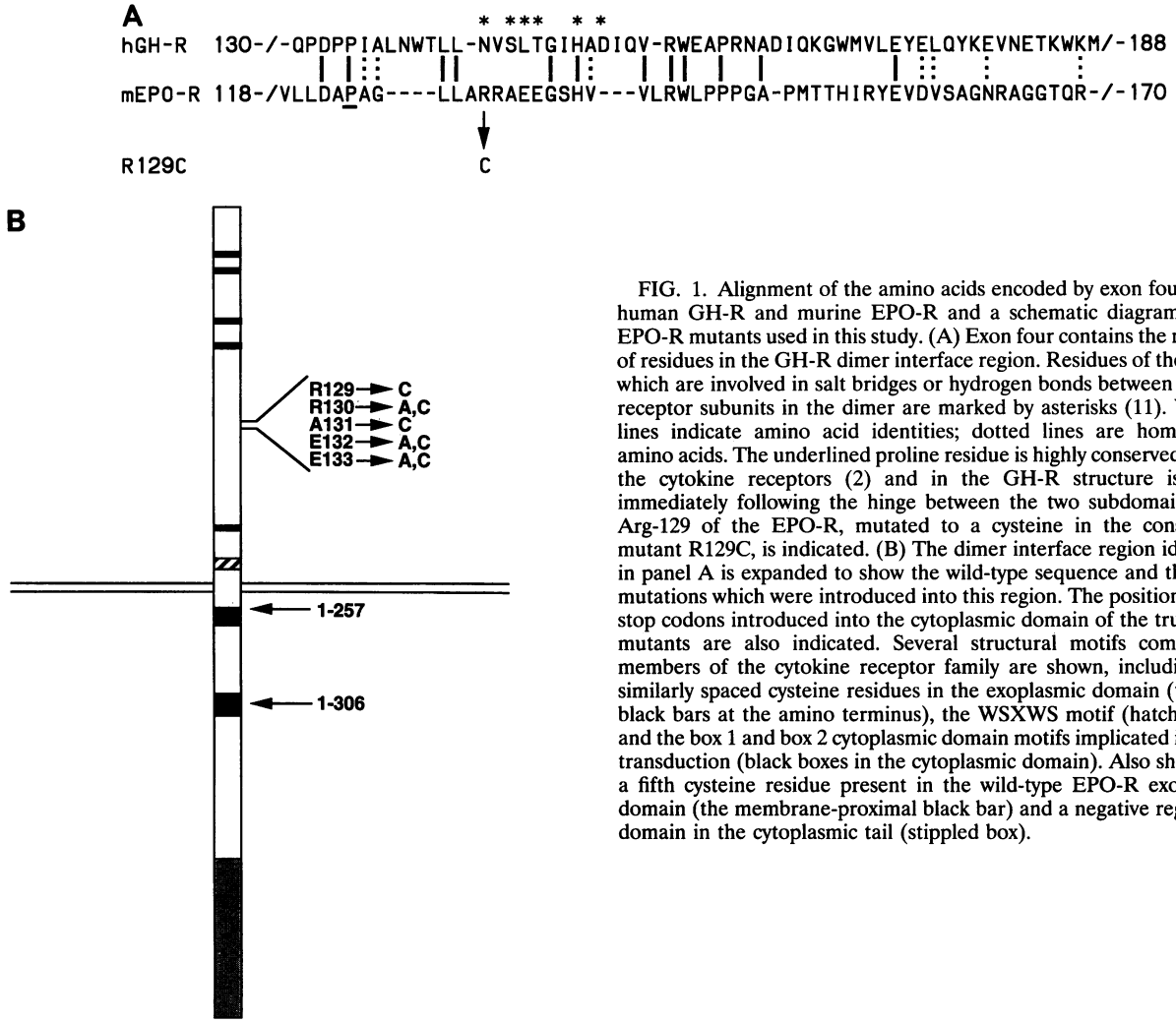


FIG. 1. Alignment of the amino acids encoded by exon four of the human GH-R and murine EPO-R and a schematic diagram of the EPO-R mutants used in this study. (A) Exon four contains the majority of residues in the GH-R dimer interface region. Residues of the GH-R which are involved in salt bridges or hydrogen bonds between the two receptor subunits in the dimer are marked by asterisks (11). Vertical lines indicate amino acid identities; dotted lines are homologous amino acids. The underlined proline residue is highly conserved among the cytokine receptors (2) and in the GH-R structure is found immediately following the hinge between the two subdomains (11). Arg-129 of the EPO-R, mutated to a cysteine in the constitutive mutant R129C, is indicated. (B) The dimer interface region identified in panel A is expanded to show the wild-type sequence and the point mutations which were introduced into this region. The positions of the stop codons introduced into the cytoplasmic domain of the truncation mutants are also indicated. Several structural motifs common to members of the cytokine receptor family are shown, including four similarly spaced cysteine residues in the exoplasmic domain (the four black bars at the amino terminus), the WSXWS motif (hatched box) and the box 1 and box 2 cytoplasmic domain motifs implicated in signal transduction (black boxes in the cytoplasmic domain). Also shown are a fifth cysteine residue present in the wild-type EPO-R exoplasmic domain (the membrane-proximal black bar) and a negative regulatory domain in the cytoplasmic tail (stippled box).

5 days of culture. Cell lines grown in EPO were cloned, and initially six clonal lines of each mutant were studied.

To determine if any of the mutants were able to confer hormone-independent growth, the clonal lines growing in EPO were transferred to medium lacking any added growth factor (see Materials and Methods for details). Untransfected 32D cells and cells expressing all but three of the mutant receptors died in the absence of any added growth factor; even after maintaining the cultures for 2 to 3 weeks with periodic feedings, no live cells were found in the cultures of cells expressing the wild-type EPO-R or mutant R130A, R130C, A131C, E132A, E133A, or AAAAA. Clones expressing E132C and E133C, however, were able to grow in the absence of added growth factor. These receptors, as well as the R129C mutant characterized previously, confer EPO-independent proliferation to transfected 32D cells (Fig. 2B and Table 1).

Unlike the clonal lines expressing the R129C mutant, the majority of cells in the clonal lines expressing the E132C and E133C mutants died when they were shifted directly from medium containing EPO to medium lacking any added growth factor. The cells that survived appeared to proliferate very slowly in the initial stages of culture. Similarly, in cultures from which EPO was removed by dilution, the cells proliferated very slowly as the concentration of EPO dropped below 0.01 U/ml.

TABLE 1. Doubling times of cell lines expressing the EPO-R dimer interface mutants

Cell line	Doubling time[a] (h)		Disulfide-linked dimerization
	EPO or IL-3	No GF	
wt[b]	10–11	NV	–
R129C	10–11	15	+
R130A	10–11	NV	–
R130C	10–11	NV	–
A131C	10–11	NV	–
E132A	10–11	NV	–
E132C	10–11	18	+
E133A	10–11	NV	–
E133C	10–11	15	+
AAAAA	10–11	NV	–

[a] Cell lines were maintained by dilution in medium supplemented with 10% FCS and 5% WEHI CM (IL-3), 10% FCS and 1 U of EPO per ml (EPO), or 10% FCS (No GF) under a concentration of 10^6 cells per ml. The cells were counted each day for up to 7 days and the doubling times were determined from the growth curves (not shown). NV, nonviable.

[b] wt, wild type.

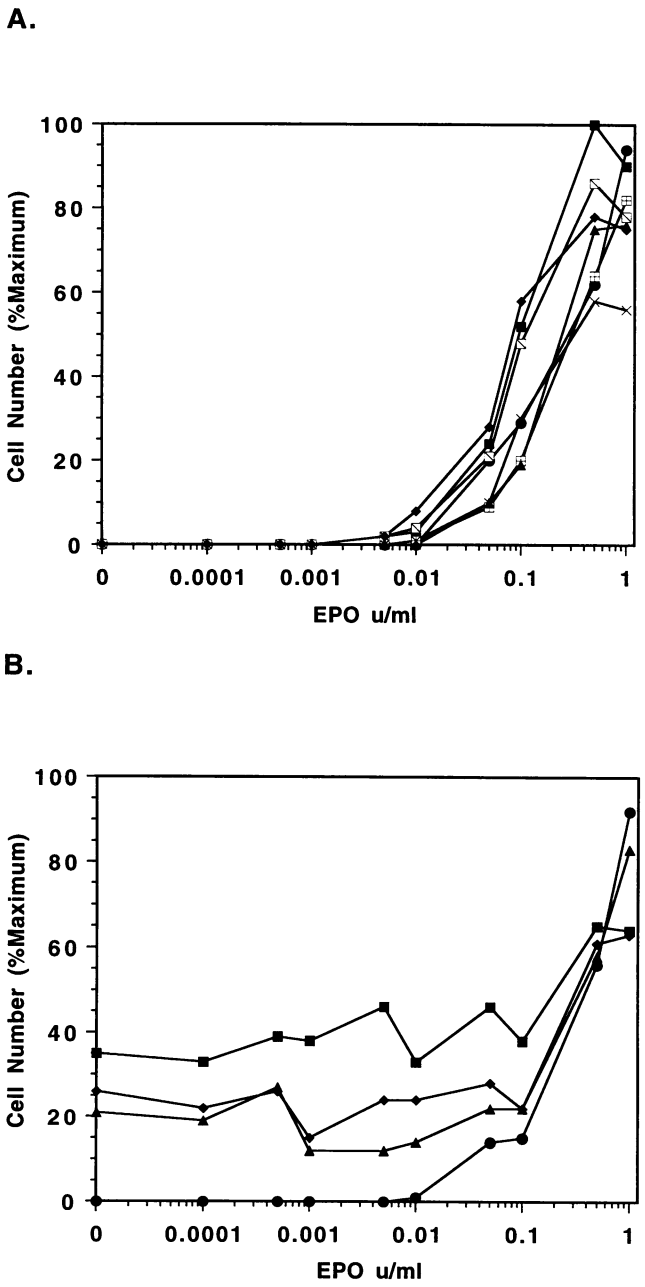


FIG. 2. Proliferation of 32D cells expressing the dimer interface mutants in various concentrations of EPO. Clonal cell lines expressing the wild-type and mutant receptors were tested for proliferation in medium supplemented with EPO (from 0.0001 to 1 U/ml) or in medium without additional growth factor (0 U/ml), as described in Materials and Methods. After 3 days in culture the cells were counted and the numbers are expressed as a percentage of the cells growing in a parallel culture in the presence of 5% WEHI CM. The symbols represent cells expressing the wild-type EPO-R (closed circles), R130A (closed squares), R130C (hatched squares), A131C (crosses), AAAAA (closed triangles), E132A (lined squares), and E133A (closed diamonds) (A) and cells expressing the wild-type EPO-R (closed circles), R129C (closed squares), E132C (closed diamonds), and E133C (closed triangles) (B).

The factor-independent clonal cell lines were maintained without supplemental growth factor in the medium for 4 to 8 weeks prior to testing in dose-response or proliferation assays; at this point in culture, the cell lines were well established and growing at a consistent rate. This adaptation phase to growth in medium lacking added growth factors may reflect selection of cells expressing higher numbers of receptors if there is a minimal number of mutant surface receptors necessary for hormone-independent proliferation.

The level of EPO-R expression varied among the 32D clonal cell lines, as assayed by immunoprecipitation of metabolically labeled receptors (data not shown). However, all six clonal lines expressing a single mutant were identical with respect to growth factor requirements, and one clone of each mutant was chosen for further study.

Cells expressing all of the EPO-dependent mutants (R130A, R130C, A131C, E132A, E133A, and AAAAA) proliferated, in the presence of EPO, at a rate similar to that of cells expressing the wild-type receptor (Fig. 2A and Table 1). The half-maximal growth response was at a concentration of approximately 0.1 U of EPO per ml (~23 pM), and the cells did not proliferate at concentrations of less than 0.001 U/ml (Fig. 2A). Thus, individual substitutions of amino acids 130 to 133 with alanine, substitution of residue 130 or 131 with cysteine, or replacement of all five residues (129 to 133) with alanine did not interfere with EPO-induced activation of the receptor. All three constitutive mutants, R129C, E132C, and E133C, proliferated in low concentrations of EPO or in the absence of EPO. However, the cells grew at a faster rate at concentrations of EPO above 0.1 U/ml (Fig. 2B). To quantify factor-dependent and -independent proliferation, the doubling times of the 32D cells expressing the mutant EPO-Rs were measured. In the presence of either IL-3 or EPO, cell lines expressing the wild-type EPO-R or any mutant EPO-R grew at approximately the same rate: ~10-h doubling time. Cell lines expressing the constitutive mutants R129C, E132C, and E133C grew at a slower rate (15- to 18-h doubling times) in the absence of growth factor, suggesting that in cells expressing these mutants either a fraction of EPO-Rs are responsive to EPO and a fraction are constitutively active or the covalently linked receptors retain some EPO responsiveness (Table 1).

Covalent dimerization of EPO-R cysteine point mutants. All mutant EPO-Rs containing substitutions to cysteine were tested for their abilities to form disulfide-linked homodimers. Preliminary studies indicated that, when expressed in 32D cells, R129C, E132C, and E133C formed disulfide-linked dimers; the level of expression of the EPO-R in these lines was, however, very low (data not shown). Thus, BA/F3 cells were transfected with cDNAs encoding the cysteine mutants and were selected for growth in EPO. Growth factor-independent proliferation was tested as described in Materials and Methods. All mutants tested behaved identically in BA/F3 and 32D cells with respect to growth factor requirements.

BA/F3 cells expressing the wild-type EPO-R and mutants R129C, R130C, A131C, E132C, and E133C were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine for 15 min and then were incubated with medium containing excess unlabeled amino acids at 18°C for a 2-h chase period. The 18°C chase allows the normally unstable EPO-R polypeptides to accumulate intracellularly. Proteins were immunoprecipitated with antiserum raised against a carboxy-terminal peptide of the EPO-R and separated in two-dimensional SDS-polyacrylamide gels in which the first dimension was nonreducing and the second was reducing. As evidenced by an ~160-kDa species under nonreducing conditions that converted to a single ~64-kDa species after reduction (Fig. 3, arrows), the constitutively

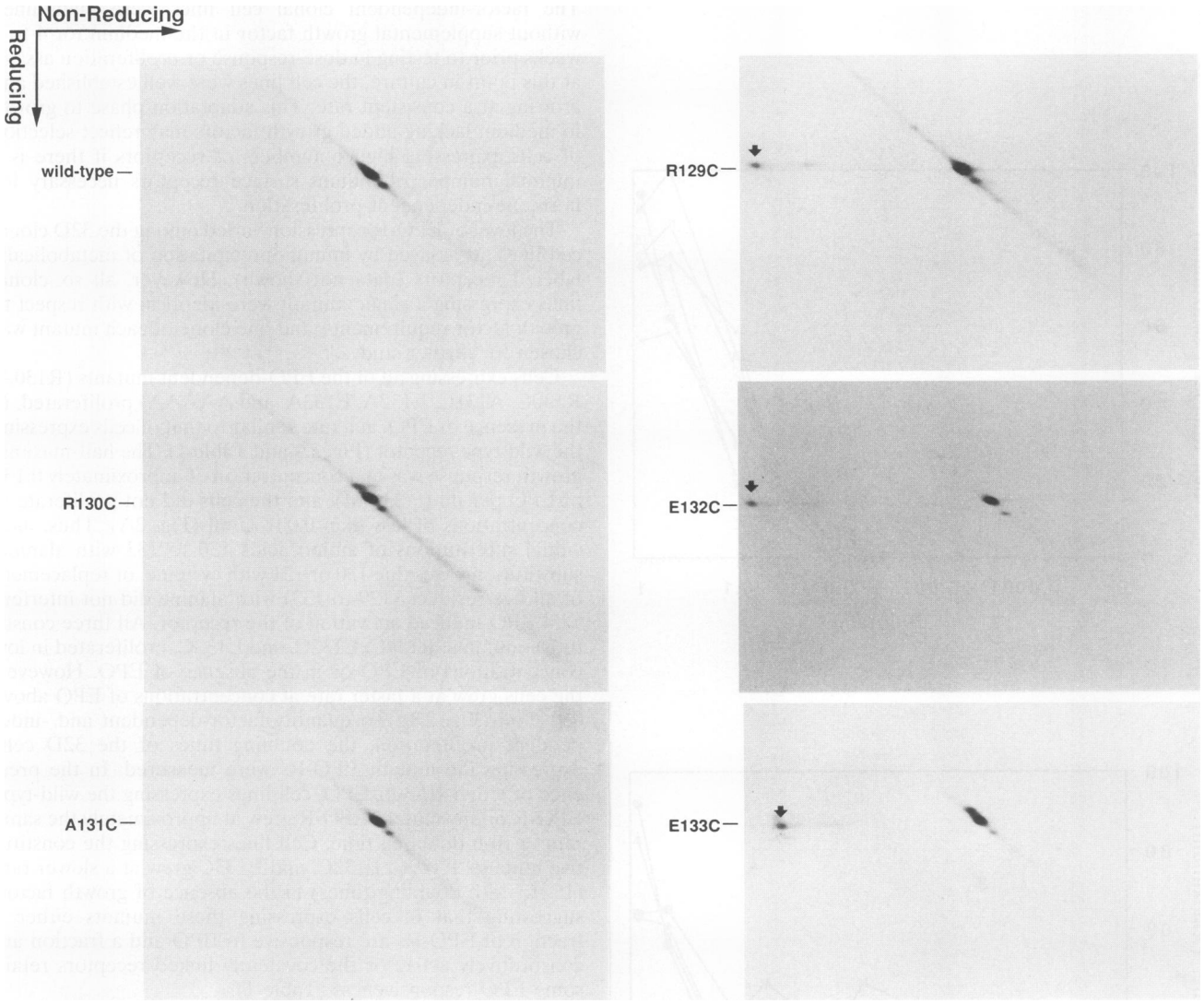


FIG. 3. Homodimerization of the constitutively active EPO-Rs. BA/F3 cells expressing the wild-type EPO-R or mutants R129C, R130C, A131C, E132C, and E133C were pulse-labeled for 15 min and chased for 2 h at 18°C in the presence of excess unlabeled amino acids. Immunoprecipitated proteins were separated by two-dimensional SDS-PAGE, and the fluorographed gels were visualized by autoradiography. The disulfide-linked dimers of R129C, E132C, and E133C (arrows) migrate identically under nonreducing conditions (~160 kDa [data not shown]); the differences detected in this experiment are due to differential swelling of the gels during fluorography.

active mutants R129C, E132C, and E133C formed disulfide-linked dimers, whereas the wild-type EPO-R and the EPO-dependent mutants R130C and A131C did not (Fig. 3). The disulfide-linked dimers migrated more slowly than expected under nonreducing conditions (~160 versus ~128 kDa); it is possible that the intermolecular disulfide linkage inhibited SDS from efficiently binding and denaturing the receptors. A minor, faster migrating form of the disulfide-linked dimers was often detected, particularly in cells expressing R129C; the identity of this species is unknown, although it may be a differentially phosphorylated or glycosylated form of the receptor.

Disulfide-linked dimers and non-disulfide-linked forms of R129C are detected on the cell surface at a ratio of approximately 1:1 (51). Cells expressing R129C display a single affinity for ^{125}I-EPO (K_d = 700 pM), indicating that both monomeric and dimeric forms of the receptor bind EPO with the same affinity (51). Preliminary evidence obtained by cross-linking ^{125}I-EPO to intact cells indicates that both monomeric and dimeric forms of E132C and E133C are also found on the cell surface, although we could not reproducibly determine the ratio of monomeric to dimeric forms. BA/F3 cells expressing R129C, E132C, and E133C receptors display a single affinity for EPO, similar to that of the wild-type receptor (K_d ~ 700 to 1,000 pM; ~1,000 binding sites per cell) (data not shown), demonstrating that covalent dimerization of E132C and E133C is not likely to significantly alter the affinity of the receptor. As found previously for the R129C mutant, cell surface expression of the E132C and E133C mutant receptors is slightly lower than that for the wild-type receptor (data not shown).

Generation of cytoplasmic truncations of the EPO-R and analysis of their biological activity. Since covalent dimerization of the EPO-R is correlated with constitutive activity, it is likely that disruption of ligand-induced receptor dimerization

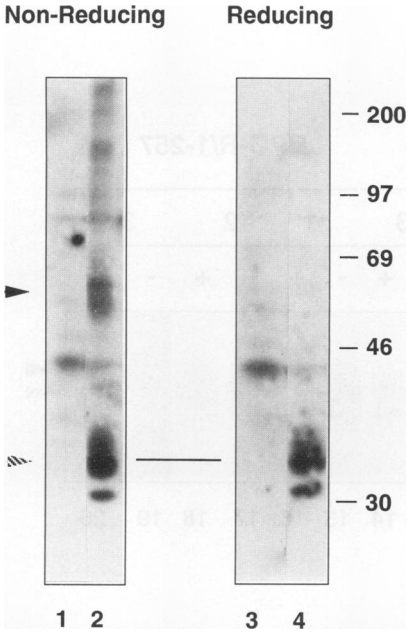


FIG. 4. Dimerization of the 1-256/R129C mutant EPO-R. Lysates from untransfected 32D cells (lanes 1 and 3) or cells expressing the 1-256/R129C mutant (lanes 2 and 4) were separated by SDS-PAGE under nonreducing (lanes 1 and 2) or reducing (lanes 3 and 4) conditions. The proteins were transferred to nitrocellulose, and the blot was incubated with an antiserum specific for the amino terminus of the EPO-R. The migration positions of the disulfide-linked dimers (~64 kDa; black arrowhead) and the monomers of 1-256/R129C (~32 kDa; striped arrowhead), as well as the migration positions of molecular mass standards (in kilodaltons), are indicated.

will repress EPO-R signal transduction. To test this hypothesis, truncation mutants of the EPO-R cytoplasmic domain (Fig. 1B) were assayed for their ability to inhibit the function of the wild-type EPO-R when coexpressed in hematopoietic cells.

Initially, plasmid DNAs encoding the truncated EPO-Rs were transfected into 32D or BA/F3 cells, and cell lines were selected for growth in medium containing IL-3 and G418. Three mutants were tested. Mutant 1-256/R129C contains nine amino acids of the EPO-R cytoplasmic tail and the R129C mutation in the exoplasmic domain. Mutants EPO-R/1-257 and EPO-R/1-306 have wild-type sequences in the exoplasmic domain and 10 and 59 residues of the cytoplasmic domain, respectively. Cell lines that synthesized EPO-R polypeptides of the predicted size (data not shown) were tested for their ability to proliferate in the presence of EPO as the sole supplemental growth factor or in the absence of supplemented growth factor. None of the mutants was able to confer either EPO-dependent or factor-independent proliferation to the transfected cells, suggesting that portions of the cytoplasmic domain required for EPO-induced proliferation had been deleted (data not shown).

Deletion of the last 40 amino acids from the cytoplasmic domain of the R129C mutant does not have an effect on disulfide-linked dimerization (51). However, the possibility that membrane-proximal cytoplasmic residues are critical for the formation of stable EPO-R dimers exists. To determine whether the membrane-proximal region of the cytoplasmic domain is required for dimerization, the mutant 1-256/R129C was tested for its ability to form disulfide-linked dimers. Disulfide-linked dimers of the 1-256/R129C mutant expressed in 32D cells were detected following electrophoresis under nonreducing conditions but not under reducing conditions (Fig. 4), demonstrating that deletion of all but nine amino acids of the EPO-R cytoplasmic domain does not have a significant effect on covalent dimerization. These results indicate that the cytoplasmically truncated EPO-Rs are likely to form ligand-induced homodimers or heterodimers if coexpressed with the full-length EPO-R.

Coexpression of the wild-type EPO-R and truncated receptors. To test the EPO-R truncation mutants for inhibitory activity, an EPO-dependent 32D cell line expressing low levels of the wild-type EPO-R was established. 32D cells were transfected with the wild-type EPO-R cDNA under the control of the adenovirus major late promoter in vector pXM, which we had previously found to be a poor promoter in these cells. Cell lines were selected by growth in EPO, and several clonal lines were isolated. One of these lines, 32Dn20, was found to have relatively low levels of EPO-R expression (data not shown) and was chosen for use in subsequent studies.

This cell line was transfected with plasmids encoding the truncated receptors, whose synthesis was driven by a strong promoter, in order to approximate a 10:1 ratio of truncated relative to full-length EPO-Rs. Such a ratio favored the detection of receptors with dominant negative activity, since the level of wild-type to truncated receptor heterodimers would exceed the level of wild-type receptor homodimers. To accomplish this, the EPO-R truncation mutants were subcloned into vector pMEX, which contains the Moloney sarcoma virus long terminal repeat as the promoter, as well as the neomycin resistance gene. 32Dn20 cells were transfected with either the vector alone or the vector encoding the EPO-R truncations, and cell lines were selected for growth in IL-3 and G418. Clonal cell lines were isolated and assayed for expression of wild-type and truncated EPO-Rs by immunoblotting of whole-cell lysates. Three clonal cell lines from each transfection were chosen for further study.

Biosynthesis of EPO-Rs in the cotransfected cell lines. To estimate the amount of truncated EPO-Rs and wild-type EPO-Rs, the coexpressing cell lines were metabolically labeled for 2.25 h and the EPO-Rs were immunoprecipitated with antiserum specific for the amino or carboxy terminus of the murine EPO-R. A portion of the immunoprecipitated polypeptides was also treated with endoglycosidase H (endo H) to assess the level of biosynthetic processing of the receptors. The results of immunoprecipitations with the carboxy-terminal specific antiserum, which reacts only with the full-length receptor, are shown in Fig. 5A. The expression level of the wild-type EPO-R in all cell lines is similar, with a maximum twofold variation. The extent of biosynthetic processing of the receptor is also similar in all cell lines (Fig. 5A). Figure 5B shows the results of immunoprecipitations with the amino-terminal antiserum, which has a higher background but which reacts with both the full-length and truncated EPO-Rs. The truncated receptors are synthesized at 5- to 10-fold-higher levels than the full-length receptor (Fig. 5B). Since the wild-type EPO-R has a half-life of ~40 min, in this experiment the cells were labeled for 2.25 h. Although we have not measured the half-life of EPO-R/1-306 and EPO-R/1-257, these labeling conditions approximate steady-state conditions, as similar ratios of full-length to truncated receptors were obtained by immunoblot analysis (data not shown).

As judged by metabolic pulse-chase experiments, the wild-type EPO-R is inefficiently transported from the endoplasmic reticulum (ER) to the medial Golgi apparatus in transfected 32D cells, with approximately 10 to 30% of the newly synthesized receptors acquiring resistance to endo H (37a). Coex-

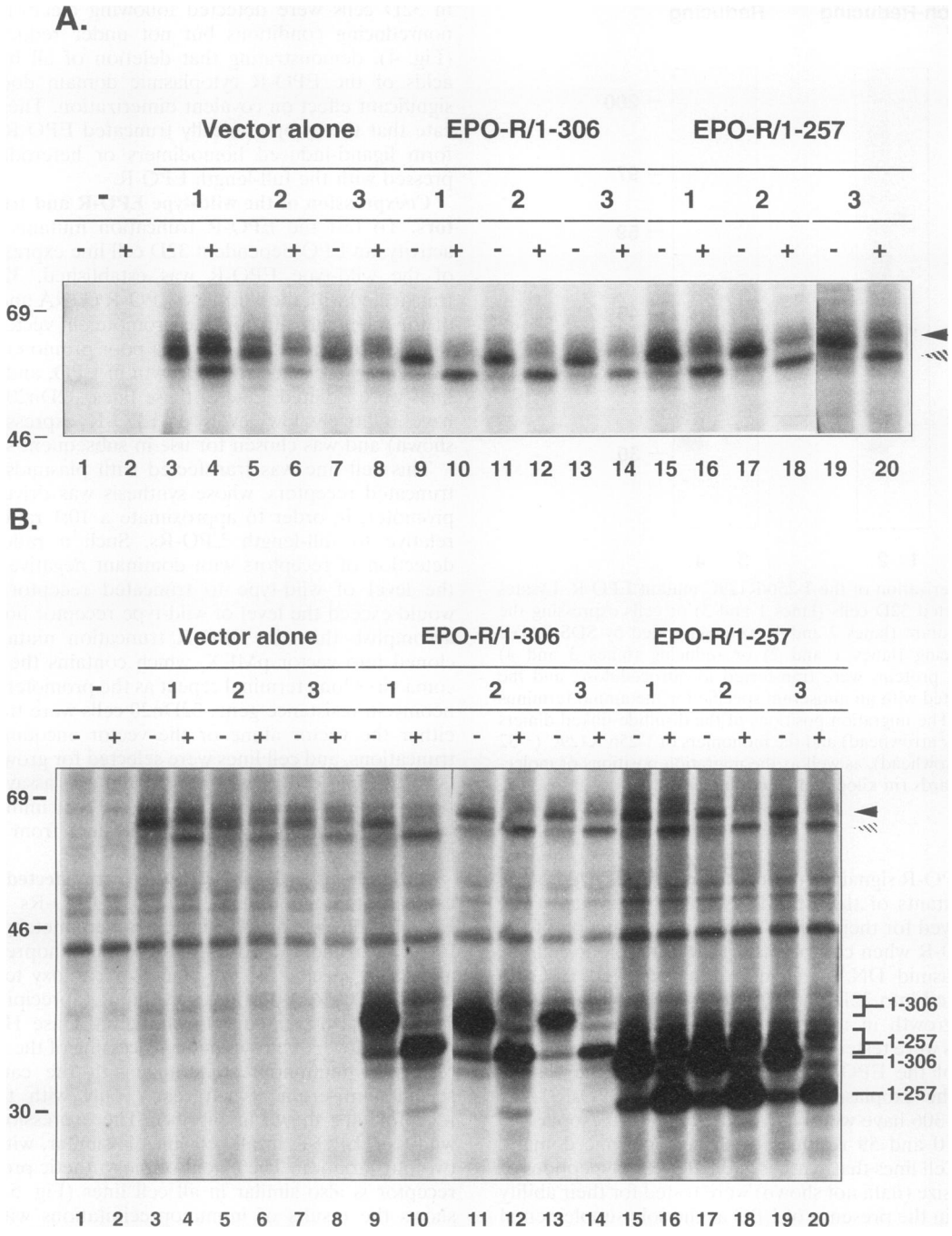


FIG. 5. Coexpression of the wild-type EPO-R and cytoplasmic truncation mutants in 32D cells. 32Dn20 cells, expressing the wild-type EPO-R, and cotransfected with the vector alone, EPO-R/1-306, or EPO-R/1-257 were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 2.25 h, and then EPO-Rs were immunoprecipitated with antiserum specific for the carboxy (A) or amino (B) terminus of the EPO-R. Three independently derived clonal lines expressing each mutant were tested in this experiment. Immunoprecipitated polypeptides were left untreated (−) or were digested with endo H (+) and resolved by SDS-PAGE. An autoradiogram of the results is shown. The migration positions of the endo H-resistant form of the wild-type EPO-R (black arrowhead) and deglycosylated, endo H-sensitive form (striped arrowhead) are indicated. Also marked are the migration positions of the deglycosylated, endo H-sensitive forms of EPO-R/1-306 and EPO-R/1-257 (bars) and the endo H-resistant species (brackets) (B). The migration positions of molecular mass standards are indicated on the left in kilodaltons.

pression of truncated EPO-Rs with the wild-type receptor in 32D cells does not have a significant effect on the amount of wild-type receptor which acquires endo H resistance (Fig. 5A), demonstrating that although the wild-type receptor is inefficiently transported out of the ER, coexpression of a vast excess of truncated receptor does not impair this transport. While there are multiple endo H-resistant species of the truncated receptors, the majority of newly synthesized molecules retain sensitivity to endo H, indicating that these forms are also poorly transported from the ER to the Golgi

TABLE 2. Cell growth and EPO-R surface expression of cell lines coexpressing the wild-type and truncated receptors[a]

Cell line and clone	% Growth in EPO	No. of EPO binding sites/cell	Affinity (pM)
Vector alone			
1	80	500	670
2	90	360	620
3	100	380	520
EPO-R/1-306			
1	60	670	780
2	33	860	590
3	45	540	700
EPO-R/1-257			
1	22	15,500	1,500
2	2	27,300	1,000
3	16	8,500	900

[a] 32Dn20 cells, expressing the wild-type EPO-R, cotransfected with the vector alone, EPO-R/1-306, or EPO-R/1-257 were used in ^{125}I-EPO equilibrium binding experiments. Three clonal lines, expressing each mutant, were tested for binding as described in the legend to Fig. 6, and the results were analyzed by the Scatchard procedure. EPO-dependent proliferation (in 100 U of EPO per ml) over a 3-day period, relative to proliferation in IL-3, was determined from the graphs presented in Fig. 7.

apparatus (Fig. 5B). The identity of the various endo H-resistant species is unclear; they may reflect forms with partial glycosylation or which differ in other posttranslational modifications.

Equilibrium binding experiments. Equilibrium binding studies with iodinated EPO were performed on cell lines coexpressing the full-length and truncated receptors to determine the effect of coexpression on receptor affinity and cell surface levels. BA/F3 or 32D cells expressing the wild-type EPO-R have very low levels of surface receptors (300 to 2,000 sites per cell). These receptors display a single affinity for EPO, with a dissociation constant in the range of 200 to 1,000 pM (51, 54). Table 2 summarizes the results obtained from binding studies on the cell lines coexpressing the full-length and truncated receptors, as analyzed by the Scatchard procedure. Representative Scatchard plots and binding curves are shown in Fig. 6. As seen in Table 2, coexpression of truncated EPO-Rs does not have a significant effect on receptor K_d values. However, surface receptor numbers are increased 2- to 50-fold in cells expressing the truncated receptors, compared with that in cells expressing only the wild-type EPO-R, suggesting that the truncated receptors are expressed at an equivalent (EPO-R/1-306 cell lines) or increased (EPO-R/1-257 cell lines) level relative to that of the wild-type receptor.

Proliferation of cotransfected cell lines in EPO reveals the dominant inhibitory activity of EPO-R truncation mutants. To determine the effect of the truncated EPO-Rs on EPO-responsive proliferation, the transfected 32Dn20 cell lines were tested for their ability to grow in concentrations of EPO ranging from 0.001 to 100 U/ml (approximately 230 fM to 23 nM) (Fig. 7). 32Dn20 cells transfected with the vector alone have an EPO response profile identical to that of 32D cells expressing the wild-type EPO-R, with half-maximal proliferation (relative to IL-3) at ~0.1 U of EPO per ml (cf. Fig. 2A and Fig. 7A). In contrast, cells expressing EPO-R/1-306 have a reduced level of proliferation in EPO, relative to their proliferation in IL-3 (Fig. 7B). The proliferative response of the cells is not stimulated significantly above ~1 to 5 U of EPO per ml, and at concentrations of EPO above 50 U/ml, over 90% of the receptor binding sites are occupied with hormone, given the K_d of the EPO-Rs (Table 2). These results indicate that a large portion of the surface full-length receptors are unable to send a proliferative signal in response to bound ligand. In addition, half-maximal stimulation is seen at a slightly higher EPO concentration, ~0.3 U of EPO per ml, than found in 32Dn20 cells transfected with vector alone (Fig. 7A and B).

Expression of EPO-R/1-257 has an even more severe effect on EPO-stimulated proliferation (Fig. 7C). The half-maximal response is at ~1 U of EPO per ml, and no significant increase in the proliferative rate is found above EPO concentrations of 10 U/ml, indicating that even above 90% receptor occupancy no additional proliferative signals are transmitted. These data also suggest that a large portion of the ligand-occupied wild-type surface EPO-Rs are unable to respond to EPO.

While the doubling times of the cells in EPO can be estimated directly from the data in Fig. 7, we chose to measure the growth rate of the cell lines directly. This independent measurement was done to determine if EPO-dependent proliferation is exponential or if an initial burst is followed by cessation of growth. The cell lines doubled at a slower rate in EPO than in IL-3 (Table 3), as predicted from Fig. 7, and followed an exponential mode of growth (data not shown). All the cell lines grew at rates similar to that of untransfected 32D cells in IL-3 (~10-h doubling time), indicating that the reduction in EPO-responsive proliferation in cells coexpressing full-length and truncated receptors is specific to EPO-mediated signal transduction and is not due to a general defect in signal transduction, DNA synthesis, or cell division.

DISCUSSION

Our results, derived from two independent experimental approaches, support the hypothesis that ligand-induced EPO-R dimerization is an essential step in signal transduction. The first series of experiments provides biochemical evidence for the role of EPO-R dimerization: EPO-Rs which form covalent disulfide-linked homodimers through cysteine residues introduced into the predicted receptor dimer interface region are constitutively active for cell proliferation in the absence of added EPO. The second series of experiments, which demonstrates that the activity of the wild-type receptor can be inhibited by coexpression with inactive, truncated EPO-R mutants, provides additional evidence for the functional role of receptor dimerization.

Disulfide-linked dimerization of EPO-R mutants correlates with their constitutive activity: mutants R129C, E132C, and E133C are all constitutively active and form disulfide-linked dimers, whereas mutants R130C and A131C are wild type with respect to EPO-dependent proliferation and fail to form detectable disulfide-linked dimers. The R129C, E132C, and E133C disulfide-linked dimers form in the ER shortly after synthesis, as judged by pulse-chase experiments (25a, 51), indicating that residues 129, 132, and 133 in neighboring receptor molecules are in proximity during receptor folding and assembly. Although residues 130 and 131 are also predicted to be in the dimer interface region (Fig. 1A), the distance between the C_α atoms in neighboring receptor molecules may be greater than 4 to 9 Å (0.4 to 0.9 nm), the distance required for disulfide bond formation, or the orientation of the side chains may not allow disulfide linkage (7).

On the basis of analogy with the GH-R, residues 129, 132, and 133 of the EPO-R, and perhaps others in proximity, may stabilize the ligand-induced homodimer (11). However, at the GH-R dimer interface, amino acids from one receptor subunit contact different amino acids in the other subunit (11), raising the possibility that either the EPO-R dimer interface region is

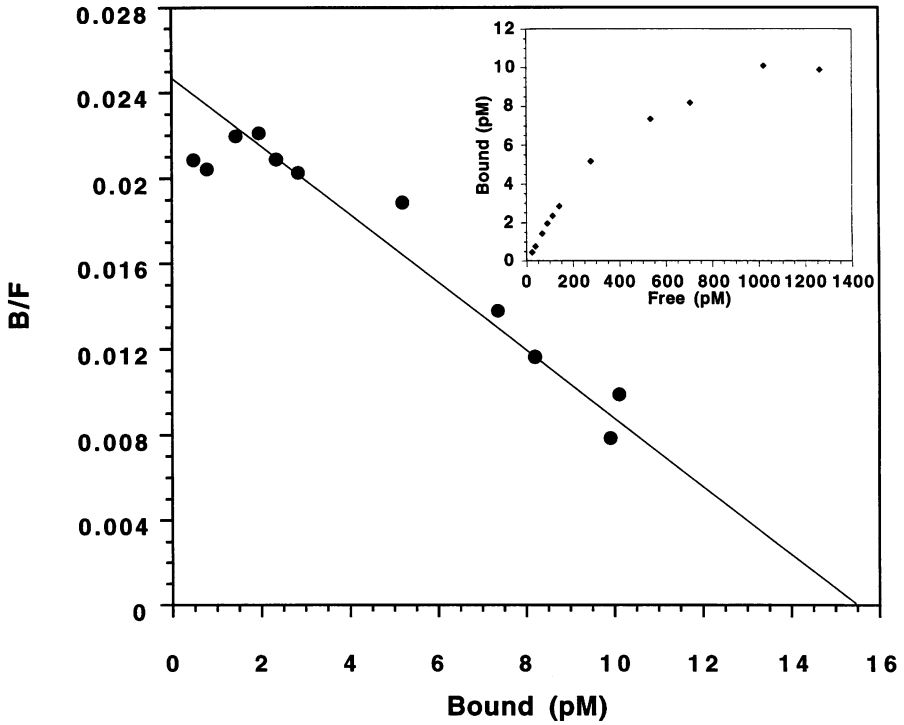


FIG. 6. Equilibrium ^{125}I-EPO binding and Scatchard analysis. 32Dn20 cells, expressing the wild-type EPO-R, cotransfected with the vector alone (A), EPO-R/1-306 (B), or EPO-R/1-257 (C) were incubated with various concentrations of ^{125}I-EPO in the presence or absence of 50 nM unlabeled EPO for 14 to 16 h at 4°C. The free and cell-bound radioactivity was counted in a gamma counter, and specific binding was determined as the difference between radioactivity bound in the absence or presence of 50 nM unlabeled competitor EPO. Scatchard plots of the data as well as the binding profiles (insets) are shown.

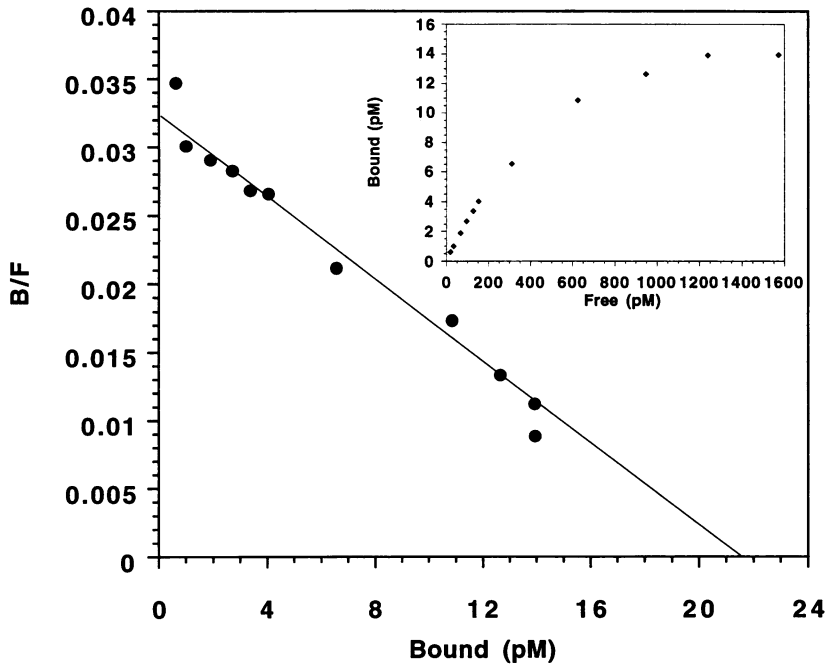
more symmetrical than that of the GH-R or the disulfide-linked EPO-R dimers are structurally different from ligand-induced dimers of the wild-type receptor. Since the R129C mutant can support EPO-independent proliferation and differentiation of murine fetal liver erythroid progenitor cells (41), it is unlikely that the disulfide-linked homodimers differ significantly in structure from the activated wild-type receptor.

Substitution of alanine for any individual residue in positions 130 to 133 or for all five residues (129 to 133) did not abolish EPO-dependent proliferation. The corresponding region in the GH–GH-R structure stabilizes the interaction between receptor subunits, but dimerization of the receptor is driven by ligand binding to a different region of the molecule (11). There are several possible explanations for why the alanine mutants are wild type in EPO responsiveness. If the EPO-R dimer interface region is slightly assymetrical, then the substituted residues may not be interacting sufficiently to destabilize ligand-driven dimerization. Since only three of the five residues we have mutagenized are close enough or in the correct orientation to form intermolecular disulfide bonds, it appears that their substitution to alanine does not significantly destabilize the dimer interface. R129C, E132C, and E133C stimulate hormone-independent proliferation; thus, it is likely the region we have mutated forms part of the dimer interface in the wild-type receptor. It is likely that we have not mapped the entire dimer interface region, since there are several other residues of the EPO-R which align with residues in the GH-R dimer interface (Fig. 1A). Identification of the residues involved in intersubunit contacts in the EPO-R dimer will be an important step towards the development of receptor antagonists and agonists, as this surface of the EPO-R molecule provides a potential target site for their binding. Saturation mutagenesis of the putative dimer interface region by cysteine substitutions provides one approach to mapping residues involved in dimerization.

Our results, as well as previously published results, suggest that disulfide-linked dimerization of the constitutive mutants mimics EPO-driven dimerization of the wild-type receptor. However, we have been unable to detect, by biochemical means, ligand-induced dimers of the wild-type receptor. Our inability to detect cell surface molecules may be due to several technical problems, including an inability to radioiodinate the low numbers of cell surface receptors (1,000 to 2,000 molecules per cell) and inefficient chemical cross-linking of receptor-receptor complexes. Even the disulfide-linked dimeric R129C receptors are difficult to cross-link with *N*-hydroxysuccinimide-based cross-linkers; this is probably related to the fact that the mature, exoplasmic domain of the receptor contains only three lysine residues (9).

In order to bypass these problems, we have pursued an

B.



C.

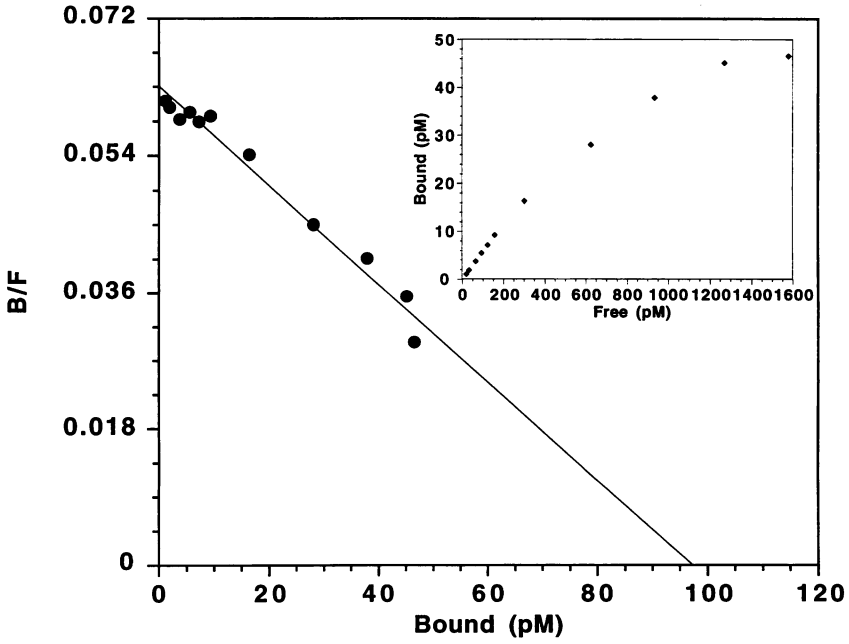


FIG. 6—*Continued.*

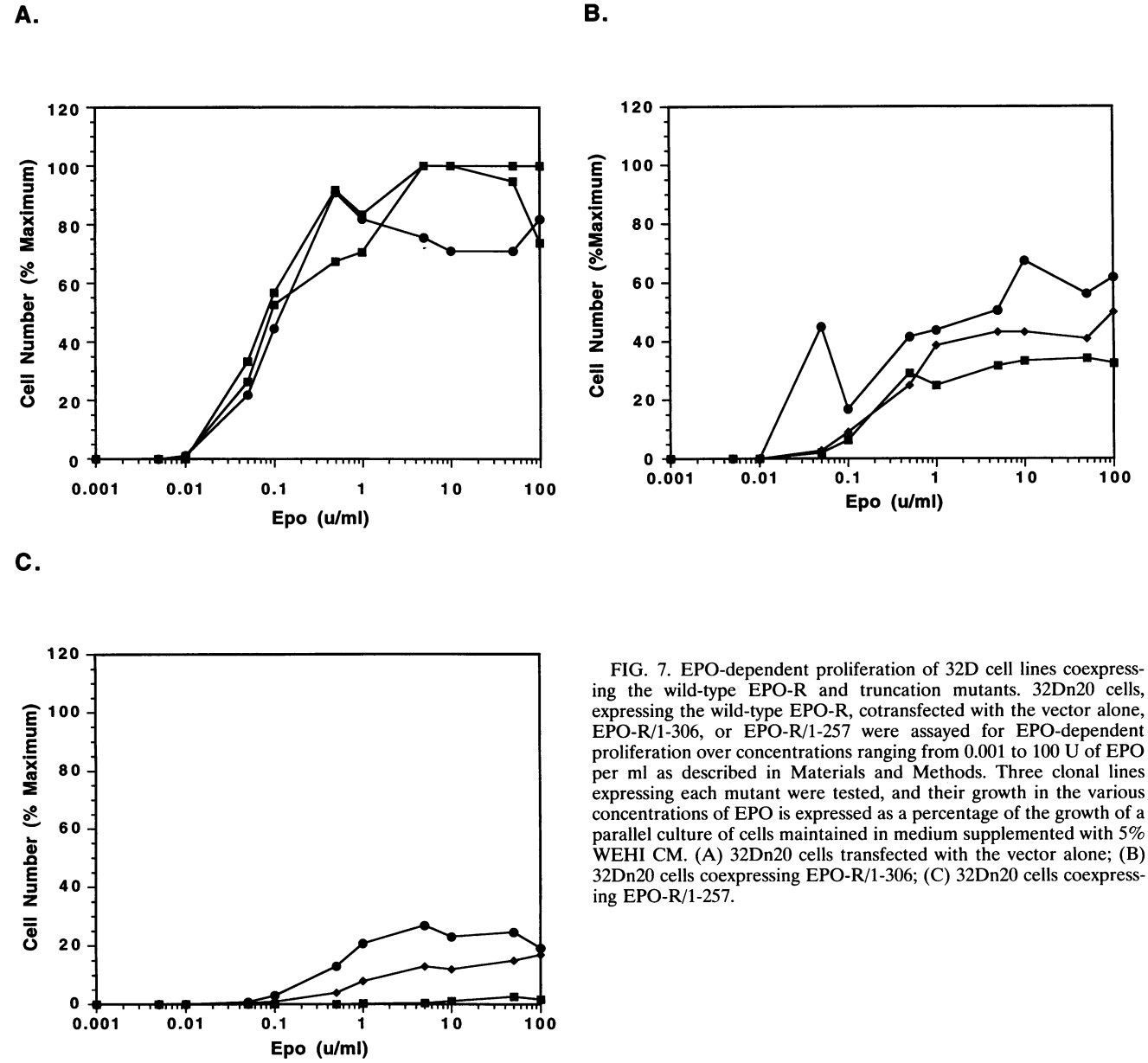


FIG. 7. EPO-dependent proliferation of 32D cell lines coexpressing the wild-type EPO-R and truncation mutants. 32Dn20 cells, expressing the wild-type EPO-R, cotransfected with the vector alone, EPO-R/1-306, or EPO-R/1-257 were assayed for EPO-dependent proliferation over concentrations ranging from 0.001 to 100 U of EPO per ml as described in Materials and Methods. Three clonal lines expressing each mutant were tested, and their growth in the various concentrations of EPO is expressed as a percentage of the growth of a parallel culture of cells maintained in medium supplemented with 5% WEHI CM. (A) 32Dn20 cells transfected with the vector alone; (B) 32Dn20 cells coexpressing EPO-R/1-306; (C) 32Dn20 cells coexpressing EPO-R/1-257.

alternative strategy. Mutant EPO-Rs, deleted for all or part of their cytoplasmic domain, were coexpressed with the wild-type EPO-R and assayed for the ability to inhibit EPO-driven proliferation. Dominant negative mutants have been reported for the EGF-R and c-Kit (3, 25, 32, 42), which are members of the tyrosine kinase receptor family, but not previously for members of the cytokine receptor family. Several independent, spontaneous mutations in the murine *W* (c-*kit*) locus have a dominant, inhibitory phenotype; these mutant c-Kit receptors have reduced kinase function (17, 38, 40, 47). Their dominant, inhibitory phenotype in heterozygous animals has been attributed to the formation of inactive complexes due to ligand-induced dimerization of wild-type and mutant receptors (32, 40). Similarly, dominant negative mutants have been generated in the EGF-R by deleting functional portions of the cytoplasmic tail. Coexpression of these deleted receptors with the full-length receptor has an inhibitory effect on signaling by the wild-type EGF-R, because of the ligand-induced formation of inactive, heterodimeric complexes (25, 42). Identification of dominant inhibitory mutants of the EPO-R provides evidence that receptor homooligomerization is required for signal transduction.

A truncated form of the R129C receptor containing only nine amino acids in the cytoplasmic tail forms disulfide-linked dimers, demonstrating that the cytoplasmic tail is not required for covalent dimerization and suggesting that EPO-R cytoplasmic domain deletion mutants will be able to dimerize with the wild-type receptor. Two truncation mutants, which by themselves cannot mediate cell proliferation, exhibit different degrees of inhibitory activity when coexpressed with the wild-type receptor. EPO-R/1-306, which has the less severe inhibitory phenotype, contains 59 amino acids of the EPO-R cytoplasmic tail, including the Box 1 motif identified in members of the cytokine receptor family and implicated in signal transduction (37). EPO-R/1-257 contains only 10 amino acids of the cyto-

TABLE 3. Doubling times of 32D cells coexpressing the wild-type EPO-R and cytoplasmic truncation mutants[a]

Cell line and clone	Doubling time (h)	
	IL-3	EPO
Vector alone		
1	10	10
2	10	10
3	9	9
EPO-R/1-306		
1	10	12
2	10	12
3	10	11
EPO-R/1-257		
1	10	14
2	11	35
3	10	18

[a] 32Dn20 cells, expressing the wild-type EPO-R, cotransfected with vector alone, EPO-R/1-306, or EPO-R/1-257 were maintained by dilution in medium supplemented with 10% FCS and 5% WEHI CM (IL-3) or 10% FCS and 10 U of EPO per ml (EPO) under a concentration of 10^6 cells per ml. Three clonal lines, expressing each mutant, were used in this assay. The cells were counted each day for 7 days, and the doubling times were determined from the growth curves (not shown).

plasmic tail and severely inhibits the ability of the wild-type EPO-R to signal cell proliferation (Fig. 7).

The reasons for the different levels of inhibition by the two truncated EPO-Rs are not clear and require further investigation. Although the EPO-R/1-306 is unable to confer EPO-dependent proliferation when expressed on its own, it may be able to interact to some degree with cytoplasmic signaling molecules, and in the presence of the wild-type receptor it may transmit a reduced, but functional, signal. Since EPO-R/1-257 lacks all but 10 amino acids of the cytoplasmic tail, it presumably cannot interact with any intracellular molecules. A comparison of cytoplasmic molecules associated with EPO-R/1-306 and those that interact with the wild-type EPO-R may be useful in determining whether the heterodimeric receptor complex is capable of a reduced level of signal transduction.

The wild-type EPO-R is rapidly degraded in the absence or presence of EPO. The majority of newly synthesized receptors are retained in the ER and are degraded with a half-life of ~40 min. Approximately 30% of the receptors synthesized in 32D cells reach the medial Golgi apparatus, as judged by the acquisition of endo H resistance; the Golgi apparatus-processed form of the EPO-R also has a half-life of ~40 min (54). The rapid degradation of the Golgi apparatus-processed form suggests that the surface EPO-Rs are internalized and transported to the lysosomes and are not recycled to the plasma membrane. An alternative explanation for the different levels of inhibitory activity between the two EPO-R truncation mutants would be a difference in their ability to be endocytosed. EPO-R/1-306 may be endocytosed more efficiently than EPO-R/1-257, leading to an accumulation of the EPO-R/1-257 mutant on the cell surface. The higher levels of EPO-R/1-257, relative to EPO-R/1-306, would favor the formation of more heterooligomeric receptor complexes in cells coexpressing the wild-type receptor and EPO-R/1-257. The results of ^{125}I-EPO binding studies suggest that EPO-R/1-257 is poorly internalized, as cell lines coexpressing the wild-type receptor and EPO-R/1-257 have ~20 to 50 times the number of surface EPO binding sites relative to cells expressing the wild-type EPO-R alone (Table 2). Experiments which directly measure the internalization rates of these mutants as well as their cell surface levels are under way and will address these questions.

Our results do not rule out the possibility that a second receptor subunit forms part of the active signaling complex. Chemical cross-linking studies on a variety of EPO-R-expressing cell lines have detected several polypeptides which cross-link to iodinated EPO (30); the identities of these species remain unresolved. Recently, using chimeric receptor molecules, Chiba and coworkers have demonstrated that chimeras containing only the extracellular portion of the EPO-R stimulate EPO-specific patterns of protein phosphorylation and gene expression associated with erythroid differentiation (4, 5). It is clear that the cell surface organization and structure of the EPO-R are poorly understood and require further investigation.

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