the following oligonucleotides; each oligonucleotide also has a 5′-TCGA-3′ Sal I extension that we added to the 5′ end to facilitate cloning, and mouse IL-2 sequences were used unless otherwise indicated: AP-1, 5′-GAAATTCCAGAGAGTCATCAGAAGA-3′; NF-IL2A, 5′-GAAAATATGTGTAATATGTAAAACATCGT-3′; IL-2κB, 5′-CCAAGAGGGATTTCACCTAAATCC-3′; NF-AT, 5′-AAGAGGAAAATTTGTTTCATACAGAAGGCG-3′; IgκB, 5′-CAGAGGGGACTTTCCGAGAGGC-3′ from the mouse κ light chain enhancer [J. W. Pierce, M. J. Lenardo, D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1482 (1988)]; human collagenase AP-1, 5′-AAGCATGAGTCAGACAC-3′ from the human collagenase promoter (*26*); and 2m, an IL-2 AP-1 oligonucleotide with mutations underlined, 5′-GAAATTCCAGAGACGAATCAGAAGA-3′. Plasmid constructions were made as follows: IL-2 (0.3 kb), a Fok I–Pst I fragment of the IL-2 promoter was cloned into the Hind III/Pst I site of pBLCAT3 [B. Luckow and G. Schutz, *Nucleic Acids Res.* **15**, 5490 (1987)]; AP-1, two copies of the oligonucleotide containing three copies of the IL-2 AP-1 site were cloned into the Sal I site of pBLCAT2 in an AA orientation; octamer, four copies of the IL-2 octamer site were inserted into pBLCAT2 in the AAAA orientation (*20*, *21*); NF-AT, three copies of the NF-AT site were cloned into the Sal I site of pBLCAT2 in the BBA orientation; IL-2κB, six copies of the IL-2κB oligonucleotide were inserted into the Sal I site of △56 such that the IL-2κB sites were in the A orientation; and IgκB, two copies of the IgκB oligonucleotide were cloned into the Sal I site of △56 to make J16. A is the orientation of the sequence in the gene, B orientation is its inversion. All plasmids were constructed with standard techniques and confirmed by sequencing. Extract (5 to 25 mg) was used in CAT assays, and we used the same amounts of protein to compare normal and anergic cells at different antigen doses. CAT conversion (percent of acetylated forms over total chloramphenicol) was quantitated with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

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34. We thank C. Chen and T. Tran for technical assistance; R. Germain for helpful suggestions; R. Bravo for antiserum to the *jun* product; D. Levens for the antiserum to the *fos* product; and D. Levens, L. Matis, U. Siebenlist, and L. Staudt for critical reading of the manuscript. Supported by a Cancer Research Institute Investigator Award (to M.J.L.) and a Howard Hughes Medical Institutes–NIH Research Scholarship (to S.-M.K.).

17 April 1992; accepted 17 June 1992

A Truncated Erythropoietin Receptor That Fails to Prevent Programmed Cell Death of Erythroid Cells

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A form of the human erythropoietin receptor (EPOR) was identified in which the cytoplasmic region is truncated by alternative splicing. The truncated form of the receptor (EPOR-T) is the most prevalent form of EPOR in early-stage erythroid progenitor cells, but the full-length EPOR (EPOR-F) becomes the most prevalent form in late-stage progenitors. EPOR-T can transduce a mitogenic signal. However, cells transfected with EPOR-T are more prone to programmed cell death than those expressing EPOR-F. EPOR-F may transduce a signal to prevent programmed cell death that is independent of the mitogenic signal, and alternative splicing of the EPOR gene may have an important role in erythropoiesis.

Erythropoietin (EPO) affects erythroid progenitors in the bone marrow by binding to the erythropoietin receptor (EPOR). The effects of EPO seem to be dependent on the stage of maturation of the cell on which it acts. EPO provides a proliferative signal to the erythroid burst-forming unit (BFU-E), early-stage progenitors, a differentiation signal to the erythroid colony-forming unit (CFU-E), late-stage progenitors (*1*), and a signal to maintain cellular viability to late-stage progenitors (*2*). EPO also inhibits DNA breakdown and prevents apoptosis in erythroid progenitor cells (*3*). Furthermore, EPO stimulates megakaryopoiesis (*4*). The mechanism by which these different signals are transmitted by one or more EPORs remains unknown.

We have detected three forms of the EPOR. Two of them were isolated by screening a cDNA library from an EPO-dependent megakaryoblastoid cell line, UT7 (*5*), with mouse EPOR cDNA as a probe (*6*). Of ten clones isolated, two had an open reading frame encoding full-length EPOR (EPOR-F) identical to the receptor isolated from liver or erythroid cell lines (*7*). Another cDNA clone had a 104-bp insert in the extracellular region (Fig. 1A). This insert introduces a stop codon before the transmembrane domain, such that this message encodes a soluble form of EPOR (EPOR-S) lacking both the transmembrane and cytoplasmic portions (Fig. 1B). The existence and the function of similar soluble receptor species have been reported for other members of the cytokine receptor superfamily (*8*).

A third form of EPOR was recovered by replicating mRNAs from normal human bone marrow (BM) mononuclear cells in the reverse transcriptase polymerase chain reaction (RT-PCR). After PCR with primers in exon I and IV (PCR 1-4) (Fig. 1A), a single 508–base pair (bp) band was observed as expected from the cDNA sequence (Fig. 1C). After PCR with the primers in exons IV and VIII (PCR 4-8) (Fig. 1A), three bands were detected. One was the 608-bp product predicted from EPOR-F; others were approximately 700 bp and 800 bp in size (Fig. 1C). We subcloned the 700-bp PCR products into pBluescript and sequenced several clones. There were two different 700-bp products with different inserts; one had a 104-bp insert (insert α) corresponding to that in EPOR-S and one contained a 95-bp insert in the cytoplasmic region (insert β) (Fig. 1A). The sequence in the 95-bp insert includes a stop codon. Thus, the message encodes an EPOR that

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lacks most of the cytoplasmic region except for 56 amino acid residues encoded by exon VII and the insert β (Fig. 1B).

The amounts of RNA encoding the alternative forms were greater than the amount encoding EPOR-F in every PCR on mRNAs from five independent BM cells (Fig. 1C) (9). On the contrary, the alternative forms were less abundant than that encoding EPOR-F in an EPO-dependent erythroleukemia cell line (JK1) (*10*) (Fig. 1C). In fact, in the cell lines UT7, K562, and KU812, EPOR-F was the dominant type (9). This may explain why EPOR-F was the first to be isolated. However, EPOR-F appears not to be the dominant form of EPOR expressed in normal BM cells.

Comparison of the sequence with that previously published (*11*) and our genomic DNA sequencing data revealed that the 104-bp insert for EPOR-S results from the presence of an imperfect splice acceptor site in the 5′ flanking region of exon V and that the 95-bp insert in EPOR-T is the remaining intron between exon VII and VIII (Fig. 1A).

We found that expression of the alternative forms of EPOR was correlated with erythroid cell differentiation. We performed RT-PCR (PCR 4–8) on mRNAs extracted from BM cells sorted in a fluorescence-activated cell sorter (FACS) according to the amount of CD34 and CD71 that they expressed. CD34 is a glycoprotein expressed only in most immature hematopoietic progenitor cells, and CD71 is the transferrin receptor that is expressed as erythroid lineage cells mature (*12*). Bone marrow cells obtained from a healthy volunteer were incubated with monoclonal antibodies to CD34 and CD71 and sorted by FACS (Fig. 2A). Ten thousand cells from each of three different fractions–$CD34^+CD71^-$ (including most immature erythroid progenitor cells), $CD71^{low}$, and $CD34^-CD71^{high}$ (most mature erythroid progenitors)—were collected. Most of the EPOR expressed in early erythroid progenitors was not EPOR-F, but as they matured there was an increase in the amount of EPOR-F (Fig. 2B). To distinguish the EPOR-S and EPOR-T products, we digested them with the restriction enzyme Pst I, which cuts only the EPOR-T PCR product into 467- and 240-bp fragments. EPOR-T was the predominant form of the EPOR in $CD34^+CD71^-$ cells, which contained about five times more EPOR-T mRNA than EPOR-F mRNA, but the $CD34^-CD71^{high}$ population contained twice as much EPOR-F mRNA as EPOR-T mRNA (Fig. 2C). The relative amount of EPOR-S was constant (approximately 20% of the amount of EPOR expressed).

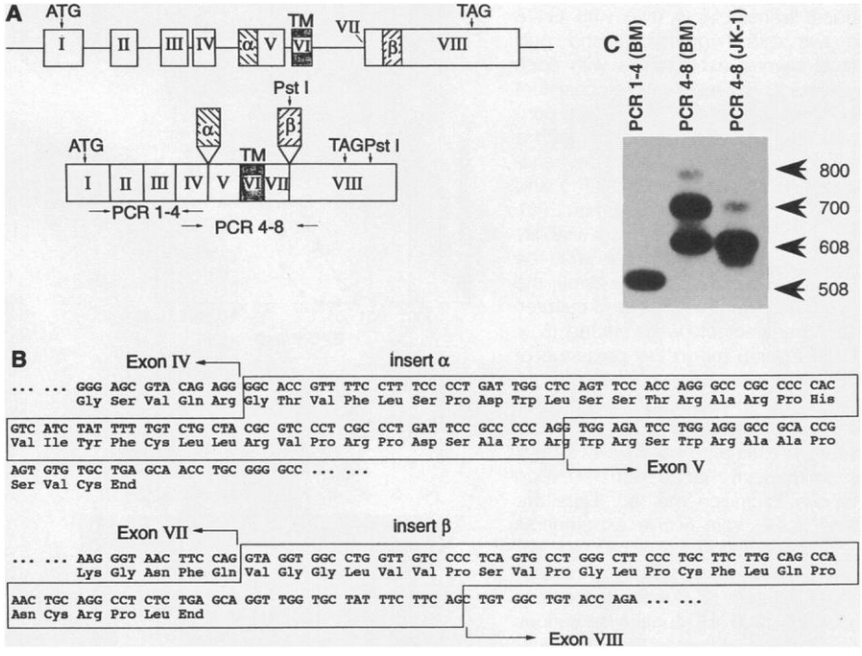

Fig. 1. Different mRNAs encoding the human erythropoietin receptor (EPOR). (**A**) Exon-intron structure of the human EPOR gene. We determined the structure by sequencing several genomic clones [obtained by polymerase chain reaction (PCR)] and comparing them with complementary DNA. Positions of the inserts α and β, ATG encoding the first methionine, the termination codon, and Pst I restriction sites are indicated. Also shown are the regions bound by primers used in PCR 1–4 and PCR 4–8. Transmembrane domain, TM. (**B**) Nucleotide sequences of the two inserts (α and β) and their flanking regions and the predicted amino acid sequences. (**C**) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of mRNA obtained from normal bone marrow cells and an EPO-dependent erythroleukemia cell line, JK1 (*10*). Reverse transcriptase reaction was performed with antisense primer 5′-TTGGATCCCTGATCATCTGC-3′ (in 3′ untranslated region). A portion (100 ng) of each sample after reverse transcriptase reaction was used for the PCR (40 cycles of denaturing at 94°C, annealing at 60°C, and polymerase reaction at 72°C). In PCR 1–4, sense 5′-AGGTCGGCTCCCTTTGTCT-3′ (in exon I) and antisense 5′-TAGCGGATGTGAGACGTCAT-3′ (in exon IV) primers were used. In PCR 4–8, sense 5′-TGAGACACCCATGACGTCTCA-3′ (in exon IV) and antisense 5′-TGTCCAGCACCAGATAGGTA-3′ (in exon VIII) primers were used. After PCR, samples were subjected to electrophoresis in an agarose gel (1.5%), and then Southern (DNA) blot analysis was performed with the Xho I–Bam H I fragment of human EPOR cDNA clone as probe. For extraction of RNA, a Micro-Fasttrack mRNA isolation kit (Invitrogen) was used.

To investigate the function of these three forms of the EPOR, we constructed an expression vector in which each form of cDNA was subcloned. Each plasmid was transfected into a mouse IL-3–dependent pro–B cell line (Ba/F3) (*13*). Cells surviving after selection with G418 (1 mg/ml) for 14 days [Ba/F3(EPOR-F), Ba/F3(EPOR-S), and Ba/F3(EPOR-T)] were assayed for EPO binding and cell growth rate. All three transfectants and mock transfectants showed the same growth and viability in the presence of mouse IL-3 (WEHI-3B culture supernatant, 10%).

Ba/F3(EPOR-S) and mock-transfected Ba/F3 cells did not bind EPO or grow in response to EPO (up to 100 U/ml) (9). However, both Ba/F3(EPOR-F) and Ba/F3(EPOR-T) cells survived in the presence of physiological concentrations of EPO (0.05 to 0.1 U/ml) and showed approximately the same single binding affinity for EPO as EPO-dependent cells (UT7) [apparent dissociation constant (K_d) for Ba/F3(EPOR-F), Ba/F3(EPOR-T), or UT7 cells ranged from 0.5 to 0.8 nM] or as Ba/F3 cells transfected with the mouse EPOR (K_d = 0.2–0.3 nM) (*14*). The number of receptors expressed in Ba/F3(EPOR-F) or Ba/F3(EPOR-T) cells ranged from approximately 4000 to 6000 per cell (9). We did not detect the higher affinity receptors reported for mouse EPOR (6) in any of these transfected cells.

The Ba/F3(EPOR-T) cells were more prone to cell death during culture. After culture in the presence of excess EPO (10 U/ml), the transfectants were cultured in medium with different concentrations of EPO. Ba/F3(EPOR-T) transfectants lost viability more quickly than Ba/F3(EPOR-F) cells at physiological concentrations of EPO (0.1 U/ml) (Fig. 3A). The difference was most obvious when EPO was not present in the culture medium.

We measured incorporation of [^{3}H]thymidine by the cells to monitor cell growth. In the presence of excess EPO, both transfectants proliferated equally (Fig. 3B). The

difference in thymidine uptake in the presence of low amounts of EPO may result from the difference in the number of viable cells at the time when [^{3}H]thymidine was added. The amino acid residues remaining in the cytoplasmic region of EPOR-T seem to be sufficient for transducing a mitogenic signal. The amino acid sequence in this region is highly conserved between mouse and human forms of the receptor (28 of 30 residues encoded by exon VII are identical).

Ba/F3 cells undergo DNA breakdown, which is characteristic of apoptosis, if mouse IL-3 is not present in the culture medium (Fig. 3C). To determine whether the death of transfectants also resulted from apoptosis, we extracted DNA from the transfectants cultured in the presence or absence of EPO and fractionated it by size on an agarose gel. More fragmentation of DNA was detected in the EPO-deprived Ba/F3(EPOR-T) cells than in Ba/F3(EPOR-F) cells. Although Ba/F3(EPOR-F) cells were viable for 24 hours in the absence of EPO (Fig. 3A), DNA breakdown did occur during this time period. However, the amount of remaining DNA of large molecular size was quite different between these two transfectants. Thus, the death of the transfected cells appears to occur by apoptosis.

Apoptosis may be inhibited by a signal mediated by amino acid residues in the cytoplasmic region that are present in EPOR-F but absent in EPOR-T. This seems more likely than a signal mediated only by EPOR-T. These data suggest that there is a signal transduction pathway that prevents apoptosis by a process independent of the regulation of mitosis. In the mouse EPOR, the most distal cytoplasmic region acts to inhibit cell growth (*14*, *15*); thus, the region mediating a signal to prevent apoptosis may be located in the middle part of the cytoplasmic region.

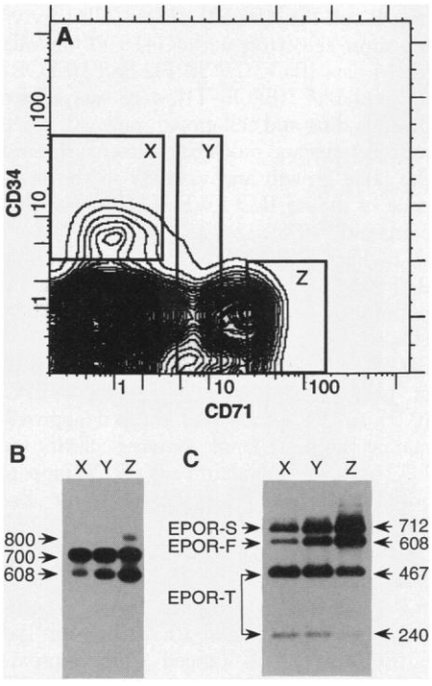

Fig. 2. Abundance of alternate forms of human EPOR during erythroid differentiation. (**A**) FACS staining profile and gating procedure for cell sorting. Bone marrow cells were stained with alophycocyanin (APC)-conjugated CD34 and fluorescein isothiocyanate (FITC)–conjugated CD71 antibodies (Becton Dickinson) (*16*). Three fractions were isolated: CD34$^+$CD71$^-$, X; CD71^{low}, Y; and CD34$^-$CD71^{high}, Z. (**B**) The mRNAs were prepared from 10,000 cells sorted from each fraction in 2A and RT-PCR (PCR 4–8) was performed. The PCR products were assayed by Southern blot analysis with a human EPOR cDNA as probe. (**C**) Each PCR product was treated with the restriction enzyme Pst I and then subjected to the same Southern blot analysis. The 700-bp EPOR-T PCR product was digested into 467-bp and 240-bp fragments with Pst I. The remaining 700-bp product represents EPOR-S (712 bp).

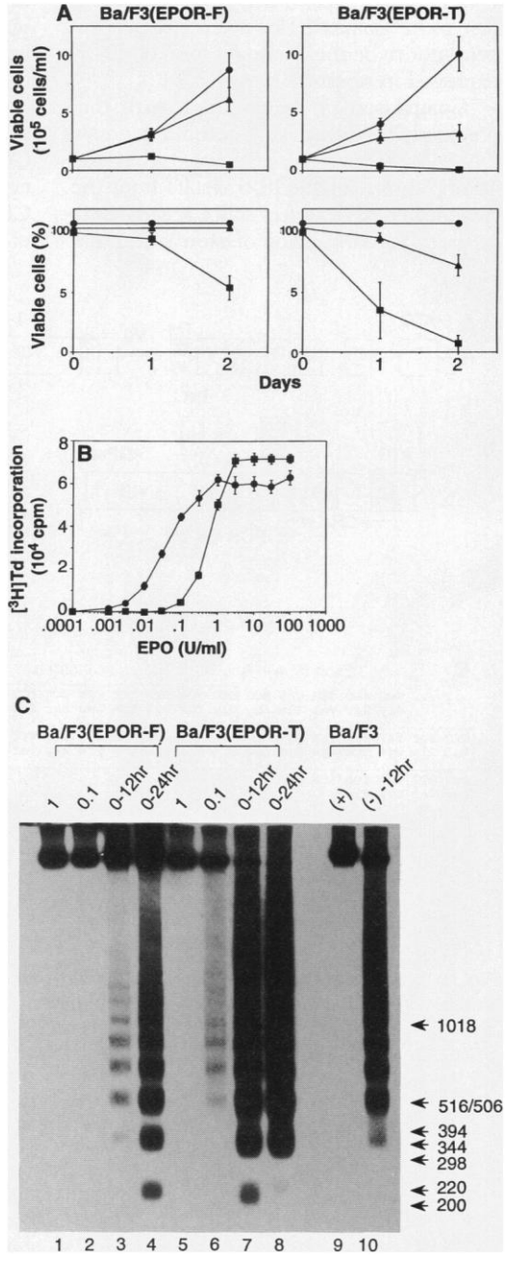

Fig. 3. Cell growth and viability studies on the Ba/F3 cells expressing different human EPORs. We constructed complete EPOR-T cDNA by substituting the Bss HII–Sph I fragment of EPOR-F with that of the PCR product from EPOR-T. Each plasmid DNA was transfected into a mouse IL-3–dependent pro–B cell line (Ba/F3) by electroporation. Two expression vectors were used; one confers resistance to neomycin (BMGneo) and the other confers resistance to hygromycin (BMGhygro). We selected four independent transfectants (two with G418 and two with hygromycin) and performed several experiments with each transfectant. The results were consistent with those shown in (A) (*19*). We confirmed the expression of EPOR with a binding assay and maintained the transfectants expressing EPOR-F and EPOR-T in the presence of human EPO. (**A**) Growth curve and cellular viability analysis. After 24 hours of culture in the presence of excess EPO (10 U/ml), the transfectants were washed and cultures of both transfectants were started (5 × 10^5 cells per 5 ml) in the presence of various concentrations of EPO (●, 10 U/ml; ▲, 0.1 U/ml; ■, 0 U/ml). Total cell number and the percentage of viable cells were estimated daily for two days (without medium change) with the trypan blue dye exclusion method. Data are means ± SD from twelve experiments with four independent transfectants. (**B**) [^{3}H]Thymidine incorporation assay. Transfected cells (10^5) were suspended in medium (200 μl) containing various concentrations of human EPO and plated in each well of a 96-well plate. The cells were cultured for 17 hours at 37°C under 5% CO_2, and then treated with 1 μCi of [^{3}H]thymidine for 7 hours. Each plot represents mean ± SD of quadruplicate experiments [●, Ba/F3(EPOR-F); ■, Ba/F3(EPOR-T)]. (**C**) Gel electrophoresis of the DNA from Ba/F3(EPOR-F), Ba/F3(EPOR-T) and Ba/F3 cells. After culture for 12 to 24 hours under different conditions, total DNA was prepared from each cell and a portion (10 μg) was subjected to agarose gel electrophoresis (1.5% gel). Southern blot analysis was performed with mouse total genomic DNA as probe. Lanes 1 and 5, DNA from cells cultured 24 hours with 1 U/ml EPO; lanes 2 and 6, 24 hours with 0.1 U/ml EPO; lanes 3 and 7, 12 hours without EPO; lanes 4 and 8, 24 hours without EPO. Lane 9, DNA from cells cultured 24 hours in the presence of mouse IL-3 (WEHI-3B cell culture supernatant); lane 10, 12 hours without mouse IL-3. The positions of size markers in base pairs are indicated.

Our observations are in accord with other reports that early- and late-stage erythroid progenitor cells differ in their responsiveness to EPO (*1*, *2*). A majority of the early-stage erythroid progenitors expressing EPOR-T may die of apoptosis (*3*), but the late-stage erythroid progenitors expressing EPOR-F may survive and differentiate into mature erythrocytes in the presence of the same physiological concentration of EPO. Thus, a mechanism exists that, under normal conditions, seemingly wastes many early-stage erythroid progenitors but helps to form a large reservoir of late-stage erythroid precursors, which can be mobilized quickly to restore erythrocyte numbers in response to sudden bleeding or hypoxia.

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16. Two-color flow cytometry analysis and cell sorting was performed on a FACSter^{plus} (Becton Dickinson) equipped with a 488-nm argon laser and a 599-nm dye laser. For controls, cells stained with APC- and FITC-conjugated monoclonal antibodies against irrelevant antigens were used. Data from 50,000 bone marrow mononuclear cells were collected. Computer-assisted data analysis of results was done on a MicroVAX computer (Digital Equipment Corp.) with FACS-DESK software (version 1.8) made available through the FACS development group at Stanford University. Residual erythrocytes and dead cells were gated out by forward-scatter and side-scatter channels, and by propidium iodide staining at the time of data collection and cell sorting.

17. We thank M. Ueda and A. Murakami for ^{125}I-labeled human EPO; K. Todokoro for cell lines; A. Shibuya for providing bone marrow samples; K. Nagayoshi for FACS operation; M. Isoda for excellent technical assistance; Y. Matsuzaki and K. Okada for secretarial help; G. Gachelin, Y. Ikawa, and Y. Miura for their support; and G. P. Nolan, S. Clark, and S. S. Jones for comments. Supported by grants from the Ministry of Education, Science, and Culture of Japan, Fujisawa Pharmaceutical Company, and Nagase Science Foundation.

10 March 1992; accepted 18 June 1992

***"There was a time when I thought the earth revolved around* her."**