Peptide Agonist of the Thrombopoietin Receptor as Potent as the Natural Cytokine

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Two families of small peptides that bind to the human thrombopoietin receptor and compete with the binding of the natural ligand thrombopoietin (TPO) were identified from recombinant peptide libraries. The sequences of these peptides were not found in the primary sequence of TPO. Screening libraries of variants of one of these families under affinity-selective conditions yielded a 14–amino acid peptide (Ile-Glu-Gly-Pro-Thr-Leu-Arg-Gln-Trp-Leu-Ala-Ala-Arg-Ala) with high affinity (dissociation constant ≈ 2 nanomolar) that stimulates the proliferation of a TPO-responsive Ba/F3 cell line with a median effective concentration (EC_{50}) of 400 nanomolar. Dimerization of this peptide by a carboxyl-terminal linkage to a lysine branch produced a compound with an EC_{50} of 100 picomolar, which was equipotent to the 332–amino acid natural cytokine in cell-based assays. The peptide dimer also stimulated the in vitro proliferation and maturation of megakaryocytes from human bone marrow cells and promoted an increase in platelet count when administered to normal mice.

Thrombopoietin is a cytokine active at many stages in the development of megakaryocytic precursors leading to the production of platelets (*1*). When injected into normal mice, TPO increases the number of megakaryocytes in the bone marrow and spleen and can increase the number of circulating platelets to more than four times the original value (*1*). Recombinant human TPO (rhTPO) is currently being tested in the clinic for the treatment of thrombocytopenia resulting from chemotherapy and bone marrow transplantation.

The cell surface receptor for thrombopoietin (c-Mpl, TPOR) is a member of the hematopoietic growth factor receptor superfamily (*2*). Extracellular domains of members of this family are typically composed of multiple β-sandwich modules related to the fibronectin type III–immunoglobulin fold, with a characteristic ligand-binding domain formed from two adjacent β-sandwich structures (*3*). The mechanism by which thrombopoietin activates TPOR is believed to be similar to that of other hematopoietic cytokines that bind and induce receptor homodimerization (*4*).

Consistent with studies showing that a small portion of the growth hormone binding site provides most of the binding energy (*5*), we recently reported the isolation of peptide mimetics of erythropoietin (EPO) from libraries of random peptides displayed on filamentous phage that bind to and activate the EPO receptor through a relatively small binding surface (*6*, *7*). In the present study, libraries of random peptides displayed as fusions to the major coat protein (pVIII) of filamentous phage or to the *Escherichia coli lac* repressor protein (peptides-on-plasmids) were used to identify peptides that bind to TPOR and act as highly potent functional mimics of thrombopoietin. The 484–amino acid extracellular domain (ECD) of TPOR was immobilized with a monoclonal antibody (mAb 179) that recognizes an epitope on the COOH-terminus of the receptor (*6*, *8*, *9*). Six pVIII phagemid libraries and three peptides-on-plasmids libraries were screened for three to four rounds against the immobilized receptor (*10*, *11*). Clones were isolated from all nine libraries and shown by enzyme-linked immunosorbent assay (ELISA) to bind specifically to TPOR (*12*, *13*). The deduced peptide sequences of 30 reactive clones are shown in Table 1.

The TPOR peptide ligands can be categorized in two families. Family 1 contains the consensus sequence VRDQIXXXL, and family 2 contains the consensus sequence TLREWL. Most of the family 2 sequences were isolated from libraries consisting of random residues flanked by a pair of cysteines, which can form intramolecular disulfide-bonded cyclic structures. Family 2 consists of two subfamilies with different spacing of the core sequence relative to the first cysteine residue and the presence or absence of the dipeptide Gly-Pro adjacent to the core consensus sequence. None of these three consensus sequences is present in the primary sequence of full-length human TPO (*14*).

We chemically synthesized a single peptide isolated from library screening that most closely represented the consensus sequence of each family and tested each peptide for competition with TPO for receptor binding. The family 1 peptide (AF12191) and the cyclic peptides from subfamilies 2A (AF12192) and 2B (AF12193) competed with TPO binding with median inhibitory concentrations (IC_{50}'s) of 20 μM to 60 nM (Fig. 1 and Table 2). The IC_{50} of rhTPO in this assay was 1 nM. A phage ELISA revealed that peptides AF12191 (family 1) and AF12192 (family 2A) blocked the binding of phage clones from families 1, 2A, and 2B, indicating that the peptide families all bind to the same site. Phage clones from each family did not bind to the extracellular domains of the related receptors for EPO, interleukin-4 (IL-4), or granulocyte colony-

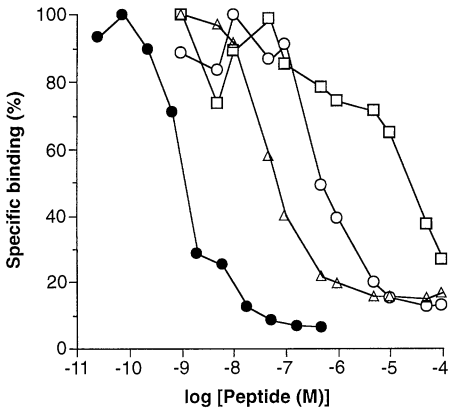

Fig. 1. Competition binding assays. A truncated form of TPO (residues 1 to 156) fused to the COOH-terminus of the maltose binding protein (MBP-TPO_{156}) was used as a tracer to measure the affinity of peptide binding to TPOR immobilized with mAb 179. Peptides were dissolved at 10 mM in DMSO and diluted in phosphate-buffered saline (PBS) containing bovine serum albumin (0.1%). Serial dilutions of peptide were mixed with a constant amount of MBP-TPO_{156} and added to TPOR-coated microtiter wells. After 2 hours at 4°C, the wells were washed with PBS and the amount of MBP-TPO_{156} bound was measured by the addition of a polyclonal antibody directed against MBP followed by an alkaline phosphatase–conjugated secondary antibody (*13*). Nonspecific binding was measured in the presence of 100 nM rhTPO (Peprotech, Rocky Hill, NJ). (□) AF12191, (○) AF12192, (△) AF12193, (●) AF12505.

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stimulating factor.

To further optimize the higher affinity family 2 sequences, we screened several mutagenesis libraries. Libraries designed to vary the residues of the core sequence (TLREWL), add random flanking residues, and substitute other amino acids for the NH_2-terminal cysteine were constructed in the headpiece dimer and polysome vectors (*15–17*). These display systems allow enrichment of ligands with higher affinity than those obtained by phage or the peptides-on-plasmids systems. The headpiece dimer library was screened against immobilized TPOR with a peptide elution technique designed to further enrich for higher affinity clones (*18*). Enriched pools were transferred to a vector that fuses the peptides to the COOH-terminus of the maltose binding protein (MBP) (*13*). The MBP-peptide fusions bind monovalently to immobilized receptor, producing an ELISA signal that correlates with the relative affinity of the free peptide (*15*).

DNA sequence analysis of clones isolated from each library revealed a highly conserved core sequence that was often flanked by charged amino acids. Some clones with a high signal in the MBP-ELISA lacked the cysteine pair (Table 2). Three peptides lacking cysteine were synthesized and had IC_{50}'s ranging from 20 to 60 nM, similar to that of the disulfide-bonded cyclic peptide AF12193. Thus, conformational constraint introduced by the disulfide bond is not absolutely required for family 2 peptides to bind with high affinity. The Gly-Pro motif found in family 2B was present in all of these linear sequences.

We then constructed a headpiece dimer mutagenesis library based on the sequence of AF12434 (IC_{50} = 20 nM), in which the core sequence (GPTLRQWL) was fixed, but flanking residues were either deleted, substituted with alanine, or conserved (*19*). Clones isolated from this library were ranked according to signal strength in an MBP-ELISA. One of the smallest peptides that retained a strong ELISA signal, IEGPTLRQWLAARA (AF12505), had an IC_{50} of 2 nM (Fig. 1)—an affinity 10 times as high as that of the parent peptide, AF12434. The first 10 residues of AF12505 are identical to the parent sequence, but the four COOH-terminal residues diverge completely from those of AF12434.

To determine if the isolated peptides activated the receptor, we constructed a TPO-responsive cell line from the murine hematopoietic progenitor cell line Ba/F3 (*20*). These cells were converted to dependence on thrombopoietin by transfection with the full-length human TPOR (*21*). The Ba/F3 hTPOR cells proliferated in the presence of rhTPO with a median effective concentration (EC_{50}) of 100 pM (Fig. 2). The high-affinity peptide AF12505 was much less potent than rhTPO with an EC_{50} of 400 nM, despite having an IC_{50} of 2 nM. Ba/F3 cells (lacking the TPOR) retained the ability to proliferate in response to IL-3,

Table 1. TPOR binding peptides isolated from random peptide libraries. Amino acid sequences of clones are grouped according to sequence homology. A consensus sequence is shown below each family (residues conserved to a lesser extent are noted below each consensus). Highly conserved residues are shown in bold, and cysteines are underlined. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Family 1	Family 2A	Family 2B
GRVRDQVAGW	CTLRQWLQGC	REGPTLRQWM
GRVKDQIAQL	CTLQEFLEGC	EGPTLRQWLA
GVRDQVSWAL	CTRTEWLHGC	ERGPFWAKAC
ESVREQVMKY	CTLREWLHGGFC	REGPRCVMWM
SVRSQISASL	CTLREWVFAGLC	CGTEGPTLSTWLDC
GVRETVYRHM	CTLRQWLILLGMC	CEQDGPTLLEWLKC
GVREVIVMHML	CTLAEFLASGVEQC	CELVGPSLMSWLTC
GRVRDQIWAAL	CSLQEFLSHGGYVC	CLTGPFVTQWLYEC
AGVRDQILIWL	CTLREFLDPTTAVC	CRAGPTLLEWLTLC
GRVRDQIMLSL	CTLKEWLVSHEVWC	CADGPTLREWISFC
GRVRDQI(X_3)L	CTLREWL(X_{2-6})C	C(X_{1-2})EGPTLREWL(X_{1-2})C
G EV	QQF	D Q M

Table 2. TPOR-binding peptides selected from random and mutagenesis peptide libraries. Synthetic peptides were tested in a TPO competition binding assay as described (Fig. 1). Cysteine-containing peptides (AF12192 and AF12193) were oxidized to the intramolecular disulfide-bonded (cyclic) form (*27*).

Library	Peptide	Sequence	IC_{50} (nM)
		Family 1	
$(X)_{11}$ pVIII	AF12191	GRVRDQIMLSLGG	20,000
		Family 2	
$C(X)_{10}C$ pVIII	AF12192	GGCTLREWLHGGFCGG	200
$C(X)_{12}C$ pVIII	AF12193	GGCADGPTLREWISFCGG	60
ON3396	AF12359	GNADGPTLRQWLEGRRPKN	60
Mutagenesis Library*	AF12434	LAIEGPTLRQWLHGNGRDT HGRVGPTLREWKTQVATKK	20
ON3410 Mutagenesis Library†	AF12405	TIKGPTLRQWLKSREHTS ISDGPTLKEWLSVTRGAS SIEGPTLREWLTSRTPHS	50
AF12434 Mutagenesis Library*	AF12505	IEGPTLRQWLAARA	2
	AF13948	IEGPTLRQWLAARA | IEGPTLRQWLAARA (β-Ala)K	0.5

*Headpiece dimer system. †Polysome display system.

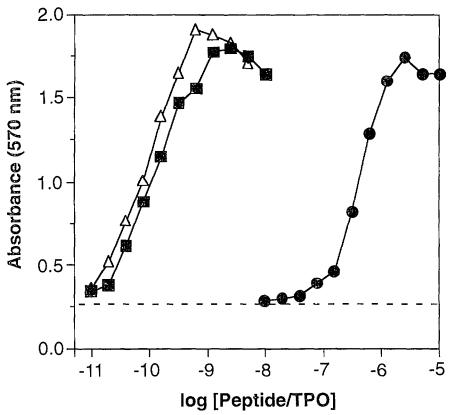

Fig. 2. Proliferative response of Ba/F3 hTPOR cells to peptides AF12505, AF13948, and rhTPO. Peptides were dissolved at 20 mM in DMSO, then serially diluted in a 96-well assay plate with an equal volume of medium [RPMI 1640 containing fetal calf serum (10%), 2 mM l-glutamine, 1× antibiotics–antimycotics]. The maximum concentration of DMSO in the assay was 0.05% (concentrations <0.5% had no effect on Ba/F3 cell proliferation). Cells were maintained in medium supplemented with WEHI-3 conditioned medium (10%) as a source of murine IL-3 (*28*). The cells were washed in PBS, resuspended in medium lacking WEHI-3, and added to each well at a density of 2×10^4 cells per well, to attain the indicated concentration of peptide or TPO. After 48 hours, proliferation was measured by a standard MTT assay (Promega). Dashed line indicates response in the absence of rhTPO or peptides. (●) AF12505, (■) AF13948, (△) rhTPO (R&D Systems).

but not in response to AF12505 or rhTPO. Ba/F3 cells transfected with the erythropoietin receptor proliferated in the presence of EPO, an EPO mimetic peptide, or IL-3, but not in the presence of TPO or the TPO mimetic peptide (*22*). A Ba/F3 line transfected with full-length murine TPOR responded to AF12505 and rhTPO with potencies similar to that of the Ba/F3 cells expressing the human receptor.

Covalently linked dimeric forms of EPO-mimetic peptides have increased potency in an EPO-dependent cell proliferation assay (*23*). To examine the effect of dimerization of the TPO-mimetic peptides on in vitro potency, we created a covalently linked dimer of AF12505. The dimer was synthesized via the α- and ε-amino groups of a COOH-terminal, β-alanine–modified lysine, creating a pseudosymmetrical dimer with respect to the α-carbon of lysine (*24*). This dimeric form of the peptide (AF13948) had an IC_{50} of 0.5 nM and stimulated Ba/F3 hTPOR cells to proliferate with an EC_{50} of 100 pM (Fig. 2), equipotent to rhTPO and more than 4000 times as potent as the monomer AF12505 from which it was derived. In a microphysiometer assay (*25*), Ba/F3 hTPOR cells responded to the same maximal level upon a 10-min exposure to either rhTPO or AF13948, with EC_{50}'s of 400 and 300 pM, respectively. Ba/F3 cells lacking TPOR showed no response to the compounds.

To assess the megakaryocyte colony-forming activity of AF13948 on cells expressing endogenous human TPOR, we cultured $CD34^+$ bone marrow cells in semisolid medium containing either rhTPO or AF13948 at 0.1 to 6 nM. After 11 days in culture, each compound produced a dose-dependent increase in the number of megakaryocyte colonies (Fig. 3A) (Spearman correlation analysis: $r = 0.67$, $P < 0.002$ for rhTPO and $r = 0.57$, $P < 0.001$ for AF13948). In the absence of rhTPO or peptide, no megakaryocyte colonies were detected.

We assessed the activity of AF13948 in promoting megakaryocyte maturation by measuring the appearance of the transmembrane glycoprotein CD41 on human $CD34^+$ cells cultured in suspension with either rhTPO or AF13948. Each compound produced an increase in the proportion of cells expressing CD41 over similar concentration ranges (Fig. 3B) (Spearman correlation analysis: $r = 0.73$, $P < 0.001$ for rhTPO and $r = 0.71$, $P < 0.001$ for AF13948), and the maximum responses were not significantly different (Students t test: $P = 0.65$).

The consensus sequences of both the TPO- and EPO-mimetic peptides contain the highly conserved dipeptide GP. The motif GPLT resides in a slightly distorted type I β turn in the bound form of the EPO-mimetic peptide, EMP-1, contributing to the global structure of the two-strand β sheet and making important contacts with the receptor. The TPO-mimetic family 2B contains the GP motif as part of the core consensus sequence GPTL, which may also induce a β turn in the peptide.

As shown for the EPO-mimetic peptide, the TPO-mimetic peptides probably activate the TPOR by inducing receptor dimerization. This supposition is supported by our observation that covalent dimerization of the high-affinity linear peptide AF12505 increases potency to 4000 times the original value. The resulting EC_{50} is 100 pM in the cell proliferation assay, essentially identical to the potency of full-length rhTPO. Thus, a small peptide, only one-tenth the size of TPO, can attain the affinity of receptor binding and potency of receptor activation possessed by the natural growth factor.

We have begun studies to examine the effects of AF13948 on thrombopoiesis in mice. Five daily subcutaneous injections of peptide at 5 μg per mouse (250 μg per kilogram of body weight, five mice per dose) increased the platelet count by 80% compared to vehicle-treated animals ($P = 0.001$). Histological examination of the bone marrow and spleen of these animals showed increased numbers of megakaryocytes, which had larger nuclear volumes but otherwise resembled the megakaryoctyes from control animals. AF13948 appears to increase platelet count by stimulating megakaryocytopoiesis and may therefore serve as a useful lead compound for the development of a therapeutically effective thrombopoietic agent.

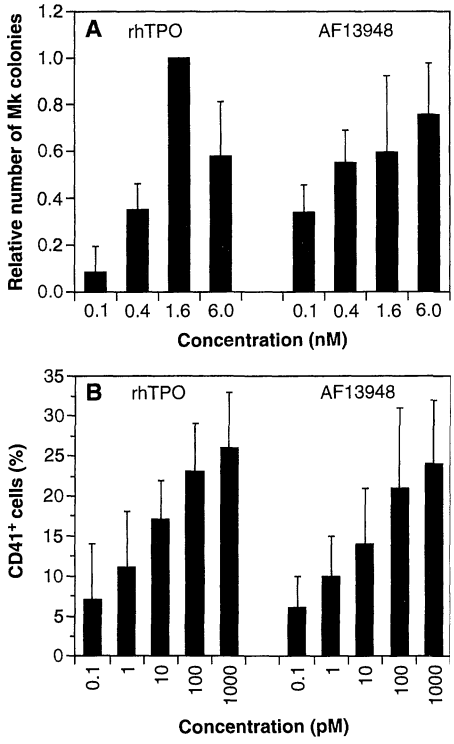

Fig. 3. Effects of rhTPO and AF13948 on human bone marrow cells. (**A**) Megakaryocyte colony formation. $CD34^+$ cells were isolated and incubated overnight at 37°C to remove adherent cells (*29*). Megakaryocyte progenitors were assayed with the MegaCult base medium kit (Stem Cell Technologies). Duplicate cultures of 8×10^3 $CD34^+$ cells in serum-free semisolid (0.2% agarose) medium containing either rhTPO (R&D Systems) or AF13948 were incubated at 37°C in 5% CO_2. After 11 days, the number of megakaryocyte (Mk) colonies containing three or more cells >25 μm were counted by light microscopy. To account for donor-to-donor variability, we calculated the relative number of Mk colonies (the ratio of colonies produced compared to the maximal rhTPO colony number in each assay) for each experiment. Values shown are the means ± SD of five separate experiments; the marrow from a different donor was used in each experiment. In the absence of rhTPO or AF13948, no colonies were detected; maximal response to rhTPO was 10 to 34 colonies. (**B**) Stimulation of human in vitro megakaryocytopoiesis. $CD34^+$ cells were cultured for 7 to 9 days in RPMI 1640 medium containing human platelet-depleted plasma (5%) and either rhTPO (R&D Systems), AF13948, or no added factors. Recovered cells were stained with a phycoerythrin-conjugated mouse antibody to human CD41 or an isotype control (Dako) and analyzed by flow cytometry (FACSort, Becton Dickinson). Bars indicate the mean percentage of $CD41^+$ cells less that in the control cultures containing no added factors ± SD from 10 individual normal donors.

REFERENCES AND NOTES

1. K. Kaushansky *et al.*, *Nature* **369**, 568 (1994).
2. I. Vigon *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5640 (1992).
3. J. F. Bazan, *ibid.* **87**, 6934 (1990).
4. W. S. Alexander, D. Metcalf, A. R. Dunn, *EMBO J.* **14**, 5569 (1995).
5. A. M. de Vos, M. Ultsch, A. A. Kossiakoff, *Science* **255**, 306 (1992); T. Clackson and J. A. Wells, *ibid.* **267**, 383 (1995).
6. N. C. Wrighton *et al.*, *ibid.* **273**, 458 (1996).
7. O. Livnah *et al.*, *ibid.*, p. 464.
8. The huTPOR ECD cDNA (*2*) was isolated from KG-1 cell mRNA with primers that allowed insertion into the Xho I and Not I sites of the α+ KH vector (*9*).
9. E. Whitehorn *et al.*, *Biotechnology* **13**, 1215 (1995).
10. Phagemid libraries displaying random peptides fused to the NH_2-terminus of pVIII were of the form $(X)_{10}$, $(X)_{11}$, $(X)_{20}$, $GGC(X)_8C$, $GGC(X)_{10}C$, and $GGC(X)_{12}C$. Libraries displaying random peptides fused to the COOH-terminus of the lac repressor protein were of the form $(X)_{15}$, $(X)_4C(X)_{4-10}C(X)_4$, and $(X)_3C(X)_{5-10}C(X)_3$. Random amino acid positions (X) were encoded by NNK, where N is an equal mixture of all four nucleotides and K is an equimolar mixture of G and T.
11. M. G. Cull, J. F. Miller, P. J. Schatz, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1865 (1992); S. D. Yanofsky *et al.*, *ibid.* **93**, 7381 (1996); C. L. Martens *et al.*, *J. Biol. Chem.* **270**, 21129 (1995).
12. R. W. Barrett *et al.*, *Anal. Biochem.* **204**, 357 (1992).
13. P. J. Schatz, M.G. Cull, E. L. Martin, C. M. Gates, *Methods Enzymol.* **267**, 171 (1996).
14. Visual inspection of the full-length human TPO se-

quence against each consensus sequence revealed no similarity. Additional comparisons with the sequences of mouse, rat, canine, or human TPO were performed with the MacDNASIS Pro (Hitachi) homology match program (based on the Lipman-Pearson algorithm). No similarity was detected with a cutoff score >20 (Ktup value, the minimum number of consecutive residues that must match in a homology region in order for a score to be assigned, was set to 2).
15. C. M. Gates, W. P. Stemmer, R. Kaptein, P. J. Schatz, *J. Mol. Biol.* **255**, 373 (1996).
16. We constructed mutagenesis library ON3396 by cloning oligonucleotides containing the sequence $(NNK)_5$ YST acK ctK cgK NaK tNK NtK $(NNK)_7$ into the headpiece dimer vector. We constructed library ON3410 by cloning oligonucleotides containing the sequence $(NNK)_4$ YST acK ctK cgK NaK tNK NtK $(NNK)_6$ TST into the polysome display system. Nucleotide abbreviations: Y = C or T; S = G or C; N = A, C, G, or T; K = G or T. Lowercase letters represent a mixture of 70% of the indicated base and 10% of each of the other three nucleotides. The degenerate codon YST encodes Cys, Ser, Pro, and Arg.
17. L. C. Mattheakis, R. R. Bhatt, W. J. Dower, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9022 (1994); L. C. Mattheakis, J. M. Dias, W. J. Dower, *Methods Enzymol.* **267**, 195 (1996).
18. Rounds 1 and 2 were performed with standard procedures (*13*, *15*). In rounds 3 and 4, 100 μM AF12191 was added to wells containing the receptor-bound plasmid complexes and incubated at 4°C for 45 min to accelerate the dissociation of lower-affinity peptides.
19. Automated "split and pool" oligonucleotide synthesis (*26*) generated a collection of oligonucleotides that encode the peptide sequence LAIEGPTLRQWLHGNGRDT in which the underlined amino acids were held constant and the remaining positions were either deleted, substituted with alanine, or conserved.
20. R. Palacios and M. Steinmetz, *Cell* **41**, 727 (1985).
21. Full-length huTPOR cDNA (628–amino acid P-form) (*2*) was isolated from KG-1 cell mRNA with primers that allowed insertion into the Xho I and EcoR I sites of the α+ vector. Expression of the receptor is under the control of the SRα promoter. Fifty micrograms of the α+ TPOR vector was electroporated into ~10^7 Ba/F3 cells. Selection was done in cell media (RPMI 1640, 10% fetal calf serum, 2 mM l-glutamine, 1× antibiotics–antimycotics) supplemented with WEHI-3 conditioned medium (10%) and G418 (1 mg/ml). G418-resistant cells were clone-sorted, and TPO-responsive clones were selected in media (RPMI 1640, 15% fetal calf serum, 2 mM l-glutamine, 1× antibiotics–antimycotics) supplemented with 1% supernatant from COS-7 cells transiently expressing full-length rhTPO.
22. D. J. Duffin *et al.*, unpublished data.
23. N. C. Wrighton *et al.*, *Blood* **88** (suppl. 1), 543a (1996).
24. The first synthesis cycle coupled 9-fluorenyl methoxycarbonyl (FMOC)–lysine (Allyloxycarbonyl protected ε-amino group) to the PAL resin (Millipore). The FMOC-protecting group was removed, followed by coupling with FMOC–β-Ala. The third cycle began with the removal of the Alloc protecting group with $[(C_6H_5)_3P]_4Pd$–4-methylmorpholine–chloroform, followed by removal of the α-amino FMOC-group. Synthesis on both branches proceeded with standard FMOC-coupling chemistry.
25. H. M. McConnell *et al.*, *Science* **257**, 1906 (1992).
26. E. A. Peters, P. J. Schatz, S. J. Johnson, W. J. Dower, *J. Bacteriol.* **176**, 4296 (1994).
27. Peptides were prepared by automated solid-phase synthesis and purified by C18 reversed-phase high-performance liquid chromatography (HPLC). Intramolecular oxidation of the thiols in cysteine-containing peptides was achieved by overnight incubation in 20% dimethyl sulfoxide (DMSO)–water, followed by purification of the cyclized peptide by reversed-phase HPLC. The structures of the purified peptides were confirmed by electrospray mass spectrometry.
28. S. Ymer *et al.*, *Nature* **317**, 255 (1985).
29. Human bone marrow aspirated from the posterior iliac crest was collected in preservative-free heparin from normal, volunteer donors with informed consent. Bone marrow mononuclear cells were isolated by density centrifugation with Ficoll-Paque 1077, washed three times with Hepes-buffered saline, and resuspended in RPMI 1640 containing 5% human platelet-depleted plasma [R. Dornsife *et al.*, *Antimicrob. Agents Chemother.* **35**, 322 (1991)]. The preparation was enriched for $CD34^+$ cells with a StemSep lineage depletion kit (StemCell Technologies, Vancouver, BC).
30. We thank E. Tate, T. Dias, Y. Feng, E. Whitehorn, and M. Bremer (receptor cloning and expression); R. Raab, G. Dawes, and C. Iverson (oligonucleotide synthesis and DNA sequencing); S. Podduturi and Q. Yin (peptide synthesis); S. Piplani, S. Johnson, and J. Dias (library screening); T. Cutler (proliferation assays) of the Affymax Research Institute; and A. Lee, I. Dev (human bone marrow assays), S. Rudolph, C. Merrell, and J. Dillberger (in vivo efficacy studies) of the Glaxo Wellcome Research Institute.

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Epilepsy and Exacerbation of Brain Injury in Mice Lacking the Glutamate Transporter GLT-1

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Extracellular levels of the excitatory neurotransmitter glutamate in the nervous system are maintained by transporters that actively remove glutamate from the extracellular space. Homozygous mice deficient in GLT-1, a widely distributed astrocytic glutamate transporter, show lethal spontaneous seizures and increased susceptibility to acute cortical injury. These effects can be attributed to elevated levels of residual glutamate in the brains of these mice.

The extracellular concentration of the excitatory neurotransmitter L-glutamate in the mammalian central nervous system must be kept low to ensure a high signal-to-noise ratio during synaptic activation and to prevent neuronal damage from excessive activation of glutamate receptors (*1*). This control is achieved by high-affinity, Na^+-dependent glutamate transporters in the plasma membrane of neurons and surrounding glial cells (*2*). The failure or reversal of these transporters may contribute to cellular damage in stroke, trauma, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease (*3*). Four subtypes of glutamate transporters have been defined by differences in sequence, pharmacology, tissue distribution, and channel-like properties: GLAST, GLT-1, EAAC1, and EAAT4 (*4*). EAAC1 and EAAT4 are selectively localized to neurons, whereas GLT-1 and GLAST are astroglial transporters (*5*). However, the roles of glutamate transporter subtypes in synaptic transmission and neurotoxicity are not known because subtype-specific inhibitors are not available. We therefore generated mice that lack GLT-1, using homologous recombination.

To disrupt the mouse gene encoding GLT-1 in E14 embryonic stem (ES) cells by homologous recombination, we constructed a targeting vector in which the exon encoding the putative third transmembrane segment was replaced with the neomycin resistance gene (Fig. 1A). Four targeted clones were identified from 144 G418- and gancyclovir (GANC)–resistant clones by Southern (DNA) blotting with 5′-flanking and 3′-flanking probes (Fig. 1A). Two mutant clones were separately injected into C57BL/6J blastocysts to produce chimeric animals. Heterozygous animals were identified by Southern blotting and were bred with each other to obtain homozygous animals, which showed the proper structure of the GLT-1 gene by Southern analysis (Fig. 1B). Northern (RNA) blotting showed that brains from homozygous mutant mice contain a hybridizable transcript that is similar in size to the wild-type GLT-1 mRNA (Fig. 1C). Reverse transcription–polymerase chain reaction experiments, followed by Southern blot analysis with an exon-specif-

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