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Discovery of a Novel Thrombopoietin Mimic Agonist Peptide

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A random phage peptide library was constructed for the filamentous bacteriophage *fuse5*. The library was made by inserting a degenerate oligonucleotide which encodes 15 variable amino acids into the NH_2-terminal region of the phage gene III protein. This library, containing 1×10^9 different phages, was screened with a human immunoglobulin fusion protein containing the extracellular region of human thrombopoietin receptor. Several phages were isolated following four cycles of enrichment and amplification. These phages specifically bound to the fusion protein. One phage peptide acted as an agonist of the thrombopoietin receptor, since it stimulated the proliferation of thrombopoietin-dependent cells and the differentiation of mouse bone marrow cells to megakaryocytes. The amino acid sequence of this peptide is not present in the primary amino acid sequence of thrombopoietin. This discovery may lead to the design of a small-molecular mimic of thrombopoietin.

Key words: megakaryocyte, peptide mimic, phage display library, synthetic peptides, thrombopoietin.

Platelets are secreted into the blood by megakaryocytes which are generated in the bone marrow by differentiation and maturation from myeloid stem cells *via* megakaryocyte precursor cells. Platelets have a lifespan of approximately 10 days in the blood, and their number is maintained at a rather constant level. The gene encoding thrombopoietin (TPO), which is a major factor controlling the hematopoiesis process by megakaryocytes, was cloned (*1-3*). TPO is a polypeptide cytokine consisting of 332 amino acids which acts as a ligand for a protein encoded by *c-mpl* (*4*), the cellular homologue of the *v-mpl* retroviral oncogene. TPO stimulates the differentiation and the maturation of megakaryocyte progenitor cells to megakaryocytes and induces proplatelet generation by binding to the TPO receptor, which is a *c-mpl* protein (MPL). TPO treatment could be useful to ameliorate thrombocytopenia, although it has significant drawbacks as a therapeutic medicine, including oral inavailability, potential immunogenicity, high cost, and other possible side effects. The polypeptidal nature of TPO underlies these drawbacks. Accordingly, one method which may overcome this problem would be to find a compound with a low molecular weight which has physiological activities similar to those of TPO.

In the past few years, several methods for the creation and screening of random phage peptide libraries have been developed (*5-7*). These libraries have been used to identify novel peptide antagonists or inhibitors for several proteins. Most recently, erythropoietin (EPO) mimic agonist peptides were discovered by the screening of random phage peptide libraries (*8*).

We report here the use of a random phage peptide library to screen for a TPO mimic peptide. We found a synthetic peptide that stimulated the proliferation of TPO-dependent cells and the differentiation of mouse bone marrow cells to megakaryocytes *in vitro*.

MATERIALS AND METHODS

Materials—Oligonucleotides were synthesized by Sawady Technology (Tokyo) and Toa Gosei (Tokyo). Peptides were synthesized by Sawady Technology. The recombinant human TPO (rhTPO), recombinant mouse stem cell factor (rmSCF), recombinant mouse interleukin-6 (rmIL-6), and recombinant mouse interleukin-11 (rmIL-11) were obtained from R&D Systems (Minneapolis, MN). The recombinant mouse interleukin-3 (rmIL-3) was obtained from Genzyme (Cambridge, MA). The recombinant mouse EPO (rmEPO) was obtained from Boehringer Mannheim (Mannheim, Germany).

Construction of Random Phage Peptide Library—The *fuse5* vector derived from fd filamentous phage (*9*) and bacterial strains were kindly provided by Prof. George Smith (University of Missouri, Columbia). A 15-amino-acid random phage peptide library was constructed following the method of Nishi *et al.* (*10*) and amplified (*9*). The 70:10:10:10 mutagenesis strategy denotes the use of a mixture of 70% correct nucleotide with 10% each of the other three nucleotides during the synthesis of the oligonucleotide used for construction of the mutagenesis library (*11*).

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Abbreviations: AChE, acetylcholinesterase; ELISA, enzyme-linked immunosorbent assay; EPO, erythropoietin; IMDM, Iscove's modified Dulbecco's medium; PBS, phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 0.73 mM KH_2PO_4, pH 7.5); rhTPO, recombinant human thrombopoietin; rmEPO, recombinant mouse erythropoietin; rmIL-3, recombinant mouse interleukin-3; rmIL-6, recombinant mouse interleukin-6; rmIL-11, recombinant mouse interleukin-11; rmSCF, recombinant mouse stem cell factor; TPO, thrombopoietin; MPL, c-mpl protein.

Purification of MPL-IgG—A chimera molecule comprising the entire extracellular domain of human MPL (amino acids 1-491) and the Fc region of human IgG (MPL-IgG) was expressed in 293 cells (*1, 12*). In brief, a cDNA fragment encoding amino acids 1-491 of human MPL was obtained by PCR of human fetal liver cDNA library (Clontech, Palo Alto, CA) and fused in-frame to a cDNA encoding the Fc region of human IgG, which was obtained by PCR of a human spleen cDNA library (Clontech). The MPL-IgG construct was subcloned into pCR3 (Invitrogen, San Diego, CA) and transfected to 293 human embryonic kidney cells by the electroporation method. The cells were selected in Geneticin (Life Technologies, Rockville, MD) at 0.4 mg/ml, and stable MPL-IgG expression was confirmed by an IgG Fc-specific enzyme-linked immunosorbent assay (ELISA). The isolated transformant was cultured to 50% confluence, and after a change of the medium to Dulbecco's modified Eagle's medium containing 1% Nutridoma (Boehringer Mannheim), the culture was continued for 7 days. After centrifugation of the conditioned medium, the supernatant was filtered. MPL-IgG was purified from the filtrate by HiTrap ProteinG column chromatography (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions.

Library Screening—MPL-IgG was immobilized at the concentration of 0.1 mg/ml in PBS (137 mM NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 0.73 mM KH_2PO_4, pH 7.5) in microtiter wells and incubated overnight at 4°C. The wells were blocked overnight at 4°C with PBS/1% BSA and washed with PBS/0.1% BSA/0.05% Tween 20. Then 1×10^{10}-1×10^{11} tetracycline-transducing units of the amplified phage library was incubated in each well at room temperature for 3 h. The wells were washed with PBS/0.1% BSA/0.05% Tween 20, and eluted with 0.1 N HCl-glycine, pH 2.2/PBS/0.1% BSA/0.05% Tween 20 at room temperature for 15 min. The eluted phage solution was neutralized and then amplified by infection into K91Kan bacteria. This process was repeated for several cycles.

Phage ELISA—MPL-IgG and human IgG (Chemicon International, Temecula, CA) were each immobilized at the concentration of 5-10 mg/ml in PBS in microtiter wells and incubated overnight at 4°C. The wells were blocked by incubation with PBS/1% BSA for 2 h at room temperature. Then 1×10^8 tetracycline-transducing units of a single phage clone was added to each well and incubated at room temperature for 2 h. The wells were washed with PBS/0.05% Tween 20, and the bound phage was detected by anti-phage ELISA according to the manufacturer's instructions (Pharmacia Biotech). The DNA sequence of the phage clones with stronger binding to MPL-IgG than to IgG was determined.

DNA Sequence Analysis—Single-strand DNA of the phage clones was isolated and the sequence of the random display insert DNA region was determined by the use of a Sequenase Version 2.0 sequencing Kit (Amersham, Buckinghamshire, England) according to the manufacturer's instructions.

Construction of BaF/mpl Cells and Proliferation Assay—Human full-length *c-mpl* P cDNA was prepared by PCR according to the published sequence (*4*) from human fetal liver cDNA (Clontech) and subcloned into pCR3. The construct was transfected into mIL-3 dependent mouse Ba/F3 cells by electroporation. The transfectants were selected in 0.8 mg/ml Geneticin and screened for the rhTPO-dependent clone BaF/mpl. For the proliferation assay, Ba/F3 and BaF/mpl cells were starved of mIL-3 by incubation in RPMI 1640 medium containing 50 μM 2-mercaptoethanol and 10% fetal bovine serum at a cell density of 5×10^5 cells/ml for 24 h at 37°C. Starved cells were plated out in 96-well plates at a density of 2.5×10^4 cells/well in the presence of various concentrations of the synthesized peptide or rhTPO (3 ng/ml; ED_{50} for BaF/mpl growth) and cultured for 24 h. For the last 6 h of culture, 0.5 mCi/well of [^{3}H]thymidine (NEN, Boston, MA) was added to each well. The cells were harvested on GF/B filters (Whatman, Maidstone, England) and washed 10 times with 0.2 ml of PBS. Then the filters were counted in the presence of 3 ml of scintillation fluid (Aquasol, NEN) in a liquid scintillation counter (Packard, Meriden, CT).

Acetylcholinesterase Assay—Balb/c mice (Charles River Laboratories, Atsugi) were killed by cervical dislocation. Bone marrow cells from the femur were prepared and treated with diisopropylfluorophosphate by the method of Burstein *et al.* (*13*). The cells were washed three times with Iscove's modified Dulbecco's medium (IMDM) and used as non-adherent bone marrow cells. The cells (5×10^5) prepared as described above were plated in a 96-well culture plate in 0.2 ml of IMDM supplemented with 1% Nutridoma in the presence of various concentrations of PK1M or hematopoietic growth factors (rhTPO, rmIL-3, rmEPO, rmSCF, rmIL-6, or rmIL-11). After incubation for 6 days at 37°C, the medium was removed, and the acetylcholinesterase (AChE) was assayed by the method of Burstein *et al.* (*13*).

Histochemical Staining for AChE—Non-adherent bone marrow cells (1×10^6) prepared as described above were set up in a LAB-TEK chamber slide (Nunc, Roskilde, Denmark) in 0.4 ml of IMDM supplemented with 1% Nutridoma in the presence of various concentrations of PK1M or rhTPO. After incubation for 6 days at 37°C, the cells were stained for AChE by the method of Burstein *et al.* (*13*). AChE-positive cells were counted with the use of a phase-contrast microscope.

Electron Microscopy—Non-adherent bone marrow cells (1×10^6) incubated with PK1M, rhTPO, or rmIL-3 for 6 days were examined by electron microscopy. They were washed with PBS at 4°C, and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.0), followed by post-fixing in 1% osmium tetroxide in the same buffer, solidified by the addition of 1% agar solution and embedded in Epon 812. The samples were cut with an ultramicrotome and stained with uranyl acetate and lead citrate for electron microscopic examination using a JEM-1200EX II electron microscope (JEOL, Tokyo). One hundred or more cells per treatment were examined ultrastructurally.

RESULTS

Construction of Random Peptide Library—A 15-amino-acid random phage peptide library was constructed for a *fuse5* vector following the method of Nishi *et al.* (*10*). This library contained 1×10^9 independent phage clones. The nucleotide sequence analysis of 38 random clones from this library indicated that inserts of 19 clones contained 15 amino acids, 14 clones contained 14 amino acids, and the remaining 5 clones contained less than 14 amino acids.

Screening of Random Phage Peptide Library—This random phage peptide library was screened for phage clones which bound to immobilized MPL-IgG. MPL-IgG is a human immunoglobulin fusion protein containing the extracellular region of human TPO receptor (*1*). After four rounds of affinity purification, it was found that phage clones were concentrated by an increase of phage recovery through the screening rounds. Phage clones that specifically bound to MPL-IgG and not to a human IgG as a negative control were selected by the phage ELISA.

A DNA sequence analysis of these phage clones revealed 6 groups of peptide sequences (Table I). Two consensus motifs were apparent in these sequences. One was GXX-LRXW between PK1 and PK2, and the other was WNXX-EF between PK7 and PK9. Not only these 6 sequences, but also these consensus motifs have no similarity to the amino acid sequence of TPO. PK1 and PK2 share another feature in that both have two cysteine residues within their peptide sequences, and thus may form an intramolecular disulfide-bonded structure.

In particular, PK1 and PK2 phage clones were obtained many times compared to other phage clones (Table I). Additionally, the phage ELISA signal of the PK1-displaying phage clone was stronger than that of the other peptide-displaying phage clones. This phage clone seemed to have a higher affinity for MPL-IgG as compared with the other phage clones.

Proliferation Assay—The peptides elicited from this random phage peptide library screening were synthesized for further characterization. Since PK1 and PK2 could be expected to have two conformations, *i.e.*, linear form or intramolecular cyclic form *via* a disulfide bond, both peptides were synthesized in two forms. To evaluate the biological potential of these peptides on TPO-dependent cells, we constructed BaF/mpl cells expressing the TPO receptor.

TABLE I. **Sequences and frequency of TPO receptor binding phage clones and phage ELISA results.**

Clone	Sequence[a]	Frequency[b]	Phage ELISA (A_{450})
PK1	LQ[G] CT[LR] A[W]RAGMC	49	0.928
PK2	CM[G] LS[LR] P[W]MLCAK	31	0.441
PK7	VR Q[WN]LT[E F]VLDTHP	1	0.160
PK9	F E[WN]YV[E F]SWASV	18	0.672
PK6	VR R QI VE Y KHRLTLP	2	0.319
PK8	ST R SESR H P F P WL L	1	0.241

[a]The amino acids are identified with a single letter code. Residues within the random peptide have been aligned to show the consensus between PK1 and 2, and PK7 and 9. [b]Shown as the number of repetitively isolated colonies which displayed the same amino acid sequence

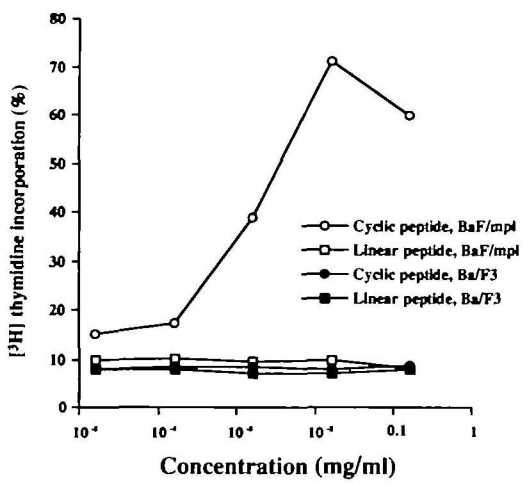

Fig. 1. **Proliferative assay of PK1 peptide.** rmIL-3-starved Ba/F3 and BaF/mpl cells were cultured for 24 h with cyclic PK1 peptide, linear PK1 peptide or rhTPO (3 ng/ml) in the absence of rmIL-3. [³H]Thymidine was added for the last 6 h, and the cells were harvested on a filter. The radioactivity on the filter was counted in a liquid scintillation counter. Data are expressed as the percentage of [³H]thymidine incorporation in cultures with rhTPO (3 ng/ml). Each value is the average of duplicate experiments.

TABLE II. **Sequences derived from the mutagenesis library.**

Clone	Sequence[a] (1 2 3 4 5 6 7 8 9 10 11 12 13 14)	Frequency[b]
PK1	L Q[G C] T[L] R A[W] R A[G] M[C]	7
PK101	L Q[G C] T[L] R A W L A[G] M[C]	43
PK102	F Q[G C] R[L] R A W R A[G] M[C]	2
PK103	I Q[G C] T[L] R A W L A[G] M[C]	11
PK104	P Q[G C] T[L] R A W L A[G] M[C]	2
PK105	L Q[G C] T[L] R A W R A[G] L[C]	2
PK106	L Q[G C] T[L] R S W R A[G] L[C]	2
PK107	L L[G C] T[L] R A W R A[G] M[C]	1
PK108	F Q[G C] T[L] R A W R A[G] L[C]	3
PK109	I E[G C] T[L] R A W L A[G] V[C]	2
PK110	L Y[G C] T[L] R A W R A[G] M[C]	1
PK111	L K[G C] T[L] R A W R A[G] V[C]	1
PK112	F Q[G C] T[L] R A W R G[G] I[C]	2
PK113	L K[G C] T[L] K A W R A[G] V[C]	1
PK114	Y H[G C] T[L] R A W R A[G] I[C]	1
PK115	F K[G C] T[L] R A W R A[G] M[C]	1
PK116	L W[G C] T[L] R V W R A[G] M[C]	1
PK117	I S[G C] T[L] R A W R A[G] I[C]	1
PK118	L R[G C] T[L] R S W R A[G] M[C]	1
PK119	F R[G C] T[L] R A[W] S A[G] I[C]	1

[a]The amino acids are identified with a single letter code. Residues within the random peptide have been aligned. The consensus amino acids are represented in enclosed boxes. [b]Shown as the number of repetitively isolated colonies which displayed the same amino acid sequence.

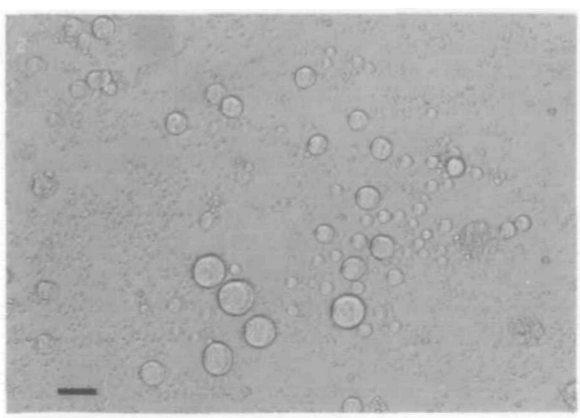

Fig. 2. **Megakaryocytes derived from mouse bone marrow cells.** Mouse bone marrow cells were incubated for 6 days with PK1M (16 μg/ml) in serum-free liquid culture. The culture was photographed under a phase-contrast microscope. The bar represents 40 μm.

We analyzed the proliferation of Ba/F3 and BaF/mpl cells with the synthesized peptides. The cyclic form of PK1 peptide stimulated the proliferation of the BaF/mpl cells, but it did not stimulate the proliferation of Ba/F3 cells in the absence of rmIL-3 (Fig. 1). Other peptides, including the linear form of PK1 peptide, did not stimulate the proliferation of either type of cells. Even at the highest concentration, cyclic PK1 peptide did not produce the same maximal proliferation response as did rhTPO; its maximal potential was approximately 71% of that of rhTPO (3 ng/ml). Further, there was a great difference in the concentrations which induced the maximal response. These results

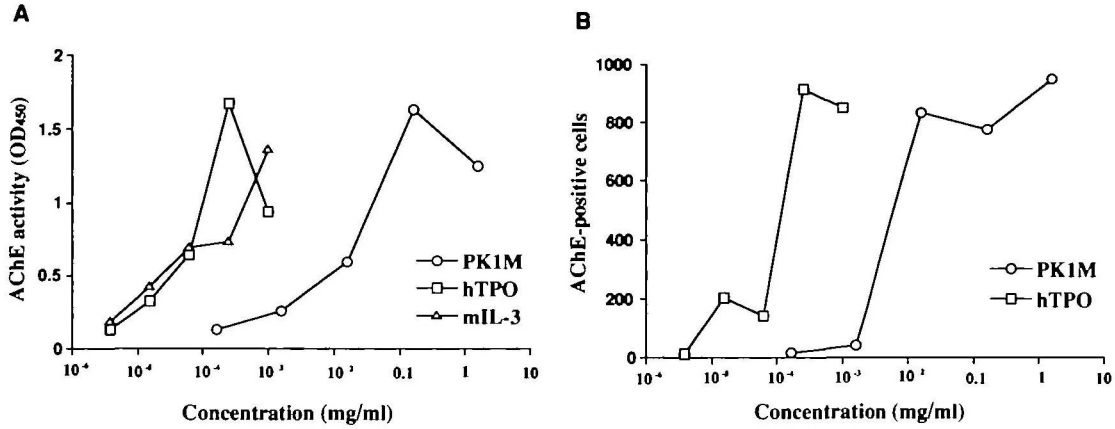

Fig. 3. **Induction of AChE in mouse bone marrow cells.** (A) Mouse bone marrow cells were cultured for 6 days with PK1M, rhTPO, or rmIL-3 in serum-free liquid culture. AChE was measured. (B) Mouse bone marrow cells were incubated for 6 days with PK1M or rhTPO in serum-free liquid culture. Cells were fixed and stained for AChE. Stained cells were counted.

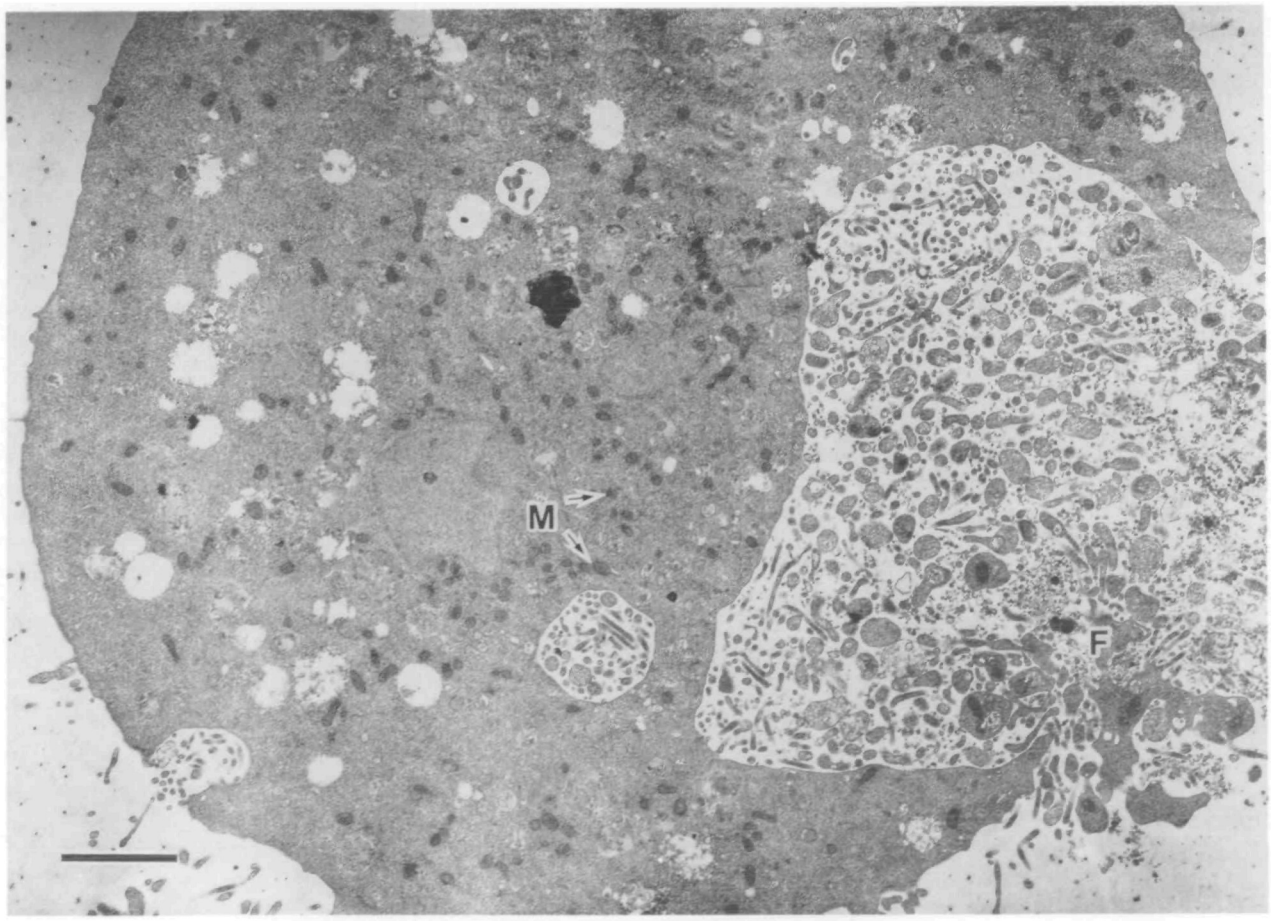

Fig. 4. **Ultrastructure of megakaryocytes derived from mouse bone marrow cells.** Mouse bone marrow cells were incubated for 6 days with PK1M in serum-free liquid culture and fixed for electron microscopy. Mitochondria (M) and fragmentation of platelets (F) were seen. The bar represents 2 μm.

indicate that, although the biological potential of cyclic PK1 peptide is weaker than that of rhTPO, it does act as an agonist to MPL.

Mutagenesis Library Screening—To determine the essential amino acids and minimum required region of PK1, we constructed a mutagenesis library. This library was constructed based on the PK1 peptide sequence such that there was a 70:10:10:10 misincorporation of each nucleotide. In theory, substitution at each codon would occur at a frequency of 34 to 50%.

This mutagenesis library was screened by the same method as described above except for the screening cycle times. After continuous second-round screening, the resultant clones were analyzed by phage ELISA. Most clones specifically bound to MPL-IgG, and based on a DNA sequence analysis, 19 kinds of peptide in addition to the original PK1 were deduced (Table II). Since all of these peptides had two cysteines at positions 4 and 14, it seemed that the cyclic form with an intramolecular disulfide bond involving the cysteines is necessary for these peptides to bind to MPL-IgG. The consensus sequence was XXGCXLX-XWXXGXC (glycine at positions 3 and 12, leucine at position 6, tryptophan at position 9). Threonine was preferred at position 5, and basic amino acids such as lysine and arginine were always at position 7. Small amino acids such as alanine and glycine were at position 11. Aliphatic amino acids were preferred at position 13.

Differentiation Assay—Since the cyclic PK1 peptide stimulated the proliferation of BaF/mpl cells, we next checked whether it would stimulate the differentiation of, and induce AChE in, mouse bone marrow cells. AChE is a marker enzyme of rodent megakaryocyte lineage cells (*14*). We found that the cyclic PK1 peptide did not induce AChE in mouse bone marrow cells. We suspected that the cyclic PK1 peptide was unstable in long-term culture, and we therefore synthesized a modified cyclic PK1 peptide (PK1M) by amidation at the COOH-terminal and acetylation at the NH_2-terminal. PK1M stimulated the proliferation of BaF/mpl cells with the same potency as that of cyclic PK1 peptide (data not shown). Bone marrow cells were cultured in the presence of PK1M or one of the following hematopoietic growth factors; rhTPO, rmIL-3, rmEPO, rmSCF, rmIL-6, and rmIL-11. In the presence of only PK1M, rhTPO, and rmIL-3, large smooth cells with cytoplasmic blebbing developed by 5 days (Fig. 2).

The AChE activity was measured in these cultured bone marrow cells. The results show that PK1M induced AChE at a higher concentration, as compared with rhTPO or rmIL-3 (Fig. 3). The maximal amount of AChE induced by PK1M was the same as that by rhTPO. The AChE-positive cells induced by PK1M and rhTPO were stained histochemically. The number of AChE-positive cells induced by PK1M was the same as that induced by rhTPO (Fig. 3).

Ultrastructural Analysis—To determine whether PK1M can complete the program of megakaryocyte differentiation, an electron microscopic study was performed. Bone marrow cells incubated with rmIL-3 were completely devoid of demarcation membranes and had few granules, results which were the same as those of a previous study (*15*). These results and those of the AChE assay show that rmIL-3 can stimulate the proliferation of bone marrow cells at least to small AChE-positive cells, but not to the immature megakaryocyte cells of Type II, as classified by Nagasawa *et al.* (*16*). In contrast, bone marrow cells incubated with PK1M or rhTPO exhibited a marked development of demarcation membranes, a mature peripheral zone devoid of organelles, and fragmentation into platelets of Type III as classified by Nagasawa *et al.* (Fig. 4).

DISCUSSION

TPO is a glycoprotein existing in at least two forms with common N-terminal amino acids (*1*). The TPO receptor is called MPL, which is a member of the hematopoietic growth factor receptor family characterized by a common structure of the extracellular domain, including four cysteines and a WSXWS motif close to the transmembrane domain (*4*). MPL seems to act as a homodimer (*17*), similar to the receptors for growth hormone (*18*), granulocyte colony-stimulating factor (*19*), and EPO (*20*). MPL-IgG seems to be homodimerized by a disulfide bond, and one group purified TPO with an MPL-IgG affinity column (*1*), so we reasoned that a TPO-mimicking low-molecular-weight peptide might be obtained by affinity screening for MPL-IgG.

The screening of random phage peptide libraries has proven to be a powerful strategy for identifying peptide ligands for a variety of target molecules. We thus tried to identify a TPO mimic from a 15-amino-acid random phage peptide library which consisted of 1×10^9 different phage clones. We obtained 6 different phage clones that specifically bound to MPL-IgG. The peptides derived from the phage clones were synthesized, and we checked whether they were TPO-mimickig agonists. One of the synthetic peptides (PK1) stimulated the proliferation of TPO-dependent cells. Modified forms of this peptide stimulated the differentiation of mouse bone marrow cells to megakaryocytes. This peptide does not show any homology to the primary amino acid sequences of human and mouse TPO (*1*). The amino acid sequence of MPL is highly conserved between human and mouse (*21*). It is thus not surprising that the PK1 peptide has agonist activity for both species.

To determine the essential amino acids of PK1, we constructed and screened a mutagenesis library based on the PK1 sequence. Since all of the peptides found retained two cysteine residues at positions 4 and 14, the cyclic form with an intramolecular disulfide bond between the cysteines seems to be necessary for these peptides to bind to MPL. The consensus sequence is GCXLXXWXXGXC.

After completion of our work, TPO receptor-binding peptides isolated from random peptide libraries were reported elsewhere (*22*). The consensus sequence of TLREWL found in that work is similar to ours.

We are interested in the structure-activity relationship of our peptides obtained by mutagenesis library screening, but unfortunately no significant increase in the activity has been obtained so far. A peptide elution technique employed by Cwirla *et al.* (*22*) might be needed to enrich active clones.

Although we obtained a TPO mimic peptide by the methods described, some problems remain. First, Cwirla *et al.* (*22*) reported that the conformational constraint introduced by the disulfide bond is not absolutely required for their TPO mimic. The Gly-Pro motif may substitute for the function of cysteines in the structure found in our study, to

expose the following amino acids to the binding site. We hope to examine this in the near future. Second, PK2, which has the same consensus motif as that of PK1, did not show any activity in our assay. We do not know what is the reason for this difference in activity, but the question may be resolved by a three-dimensional nuclear magnetic resonance or X-ray crystal analysis. A third question is whether the PK1 dimer peptide has more potential activity than its monomer, as claimed for the TPO mimic peptides (*22*). A crystal structure analysis of the peptides and MPL-IgG complex may clarify these issues. There is a possibility that other peptides which have more potent biological activity than the PK1 cyclic peptide will be found, since the diversity of our library was 10^9 clones, far less than the theoretically calculated number of combinations of 15 amino acids, which is 3.3×10^{19}.

Although low-molecular-weight mimics of peptides, such as cholecystokinin A (*23*) and somatostatin (*24*), have been discovered, non-peptide mimics of high-molecular-weight growth factors or cytokines have not yet been found. The present discovery of a TPO mimic peptide may lead to the identification of a low-molecular-weight non-peptide TPO mimic in the near future.

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