Radiodinated erythropoietin (Epo) was bound specifically to the cells of two non-erythroid clonal lines, PC12 and SN6, which expressed neuronal characteristics. The binding was time-, cell number-, and dose-dependent and was reversible. Although the cloned Epo receptor from PC12 cells (derived from rat adrenal medulla) was identical to that from rat erythroid cells, significant differences in the ligand binding properties between two cell lineages were found: 1) PC12 cells had a single class of binding sites with very low affinity ($K_d = 16 \text{ nM}$), whereas erythroid cells had two classes of binding sites with different affinities ($K_d = 95 \text{ pm} \text{ for high affinity sites and } 1.9 \text{ nM for low affinity sites}$), and 2) cross-linking experiments revealed one cross-linked product of 105 kDa for PC12 cells and two products of 140 and 120 kDa for erythroid cells. Taken together with additional results, the presence of a putative accessory protein(s) that may alter the ligand binding affinity through interaction with Epo receptor is discussed. The binding of Epo to PC12 cells caused a rapid increase in the cytosolic concentration of free calcium. The presence of EGTA had no effect on the Epo binding but completely inhibited the calcium increase, indicating that Epo stimulated the calcium influx from outside of the cells. The addition of Epo to the culture media of PC12 cells elevated the intracellular concentrations of monoamines.

Epo is a major physiological regulator of erythropoiesis.

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The abbreviations used are: Epo, erythropoietin; Epo-R, erythropoietin receptor; NGF, nerve growth factor; HuEpo, human erythropoietin; rHuEpo, recombinant human erythropoietin; DMEM, Dulbecco’s modified Eagle’s medium; MEM, minimum essential medium; PBS, phosphate-buffered saline; FCS, fetal calf serum; CHAPS, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; APMSF, (p-amidinophenyl)mercuriethanesulfonate fluoride; IL, interleukin; GM-CSF, granulocyte/macrophage-colony-stimulating factor; RT-PCR, reverse transcriptase-polymerase chain reaction; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; MDOPA, 3-methoxy-DOPA.

The action of Epo on erythroid precursor cells has been only a generally accepted function; Epo supports survival of the cells and stimulates their proliferation and differentiation (see Ref. 1 for review). Epo, however, acts in vitro on other cells besides erythroid cells; Epo promotes differentiation of megakaryocytes (2), has a mitogenic and positive chemotactic effect on endothelial cells (3), and enhances the immunoglobulin production by B lymphocytes and their proliferation (4). Epo-R is present in the cells from rodent placentas (5). A physiological significance of these findings remains to be proven but such findings of non-erythroid cells bearing Epo receptors may provide us with an opportunity to find a new physiological function of Epo.

Cells derived from the neural crest lineage appear to display in culture either neuronal or chromaffin phenotypes. NGF induces neuronal characteristics and corticosteroids potentiate chromaffin properties (6). The rat cell line PC12, which has been established from an adrenal medullary pheochromocytoma, has similar bipotent properties; the cells express more neuronal properties with exposure to NGF and keep chromaffin properties with corticosteroids (7–9). The cell line SN6, a clonal hybrid cell line developed from the septal region of the mouse basal forebrain, expresses characteristics typical of cholinergic neurons (10). Here we report the presence of Epo-R in these two cell lines, molecular properties of Epo-R on PC12 cells compared with that on erythroid cells, and the Epo-induced increase in intracellular concentrations of calcium and monoamines of PC12 cells. The presence of Epo-R on these neural cell lines can be rationalized on the basis of recent findings that Epo augments choline acetyltransferase activity in primary cultured neurons and supports the in vivo survival of lesioned neurons (11), although the physiological significance of these findings has not been verified.

Comparison of Epo-R on the neural cells with that on erythroid cells, therefore, is important for studying a new physiological function of Epo and also for understanding the Epo-induced signal transduction pathway, including identification of protein(s) on neural cells involved in the interaction with Epo.
**Erythropoietin Receptor on Cells with Neural Properties**

3, Collaborative Research, Inc.; human IL-6; tumor necrosis factor and GM-CSF, Genzyme; human EDF (activin A), a kind gift from Dr. Higashio of 3, Collaborative Research, Inc.; human IL-6; tumor necrosis factor and R. Sasaki, manuscript in preparation.

**Preparation of HU-EPO-fixed Gel**—For preparation of the Epo-fixed gel, 150 mg of rHuEpo in 100 mM NaHCO₃ was gently mixed with 9 mL of CH-Sepharose 4B gel at 20 °C for 1 h and at 4 °C overnight. The gel was pelleted by centrifugation and then suspended in 100 mM Tris-HCl, pH 8.0, to block the remaining sites on the gel. The gel was then mixed with each of the acid-containing solutions (50 mM acetic acid containing 500 mM NaCl) and then with the basic solution (50 mM Tris containing 500 mM NaCl). The Epo-fixed gel was kept in PBS containing 0.1% NaN₃ at 4 °C before use.

**Immunological Detection of Epo-R Solubilized from PC12 Cells and Rat Erythroid Cells by Western Blotting**—Epo-R solubilized from PC12 cells and rat spleen erythroid cells was concentrated using the Epo-fixed gel and then identified with the Western blotting technique. About 10⁶ cells were lysed by incubation at 4 °C for 1 h in 2 ml buffer A, PBS containing 0.5% (w/v) CHAPS, 10 μM α-APMSF, 10 μM leupeptin, and 1 mM EDTA. The lysate was centrifuged 12,000 × g for 5 min and the supernatant was discarded. The pellets were suspended in ice-cold 0.25 M acetic acid, but the inter- nalized Epo-R was pelleted by centrifugation at 4 °C. The pellets were dissolved in 30 μl of 1% SDS-buffer containing peroxidase-fixed goat anti-rHuEpo, and no NaN₃ were incubated at 15 °C for 3 h or at 37 °C for 1 h. The cell-associated ligand at 15 °C is equivalent to the cell surface receptor-bound ligand, whereas at 37 °C it contains both the receptor-bound ligand and the internalized ligand. Cells were washed three times with PBS and then suspended in ice-cold 0.25 M acetic acid, pH 2.5, containing 0.5 M NaCl or in PBS. The receptor-bound Epo should be released upon incubation at the acidic pH, but the internalized Epo should remain cell-associated. The cell suspensions were incubated at 0 °C for 3 min and layered on the cushion buffer. After centrifugation, the tube contents were frozen, and the tips were cut off as described previously. Radioactivity of the tips represents the internalized Epo and that of the supernatant represents Epo released from Epo-receptor complexes on the cell surface. Control runs were done in the presence of 200-fold unlabeled Epo.

**Cross-linked Products**—Cross-linked products between ¹²⁵I-rHuEpo and Epo-R were prepared as described previously (18). Briefly, cells (1.5 × 10⁶) were incubated with 6 nM ¹²⁵I-rHuEpo at 4 °C overnight. The cells were washed and then incubated for cross-linking in 4% glutaraldehyde solution for 1 h. Cross-linked products were extracted by the extraction buffer containing 2% Triton X-100, 10 μM APMSF, 10 μM pepstatin, 10 μM leupeptin, and 1 mM EDTA and then analyzed by SDS-polyacrylamide gel electrophoresis.

**Anti-mouse Epo-R Antiserum**—Recombinant mouse soluble Epo-

R lacking cytoplasmic and transmembrane domains was produced and isolated (22). Rabbit anti-mouse soluble Epo-R antiserum was prepared by injection of the isolated soluble Epo-R. Rabbit anti-NH₂-terminal mouse Epo-R antiserum was produced using the 15 NH₂-terminal amino acid peptide conjugated to keyhole limpet hemocyanin as an antigen (22).

**Calcium Concentration**—Intracellular calcium concentrations were determined by the direct sequencing method (27) of cloned RT-PCR products by the dye-exchange method using Sequences (28).

Northern Analysis of Epo-R mRNA from PC12 Cells and Erythroid Cells—Total RNA from PC12 cells was prepared using the method for preparation of cytoplasmic RNA (24). Total RNA from anemic rat spleen and fetal mouse liver cells was prepared according to the manufacturer's instructions (Pharmacia) using oligo(dT)-Latex. Poly(A)+ RNA (8 pg) treated with glyoxal/dimethyl sulfoxide (24) was loaded in each lane of a 1% agarose gel. Polyacrylamide gel was immersed in 15 ml of block ace at 4 °C overnight for blocking. The filter was then dipped into 15 ml of buffer B consisting of 0.05% Tween 20, 5% block ace in PBS, and the antibody of interest in 15 ml of buffer C containing 0.01% bromophenol blue. Solubilized proteins were separated by electrophoresis with SDS, 5% polyacrylamide gel. Western blotting was carried out according to the method of Burnette (23) with some modifications. Briefly, polyacrylamide gel was immersed in 50 ml of transfer buffer consisting of 48 mM Tris, 39 mM glycine, 1.3 mM SDS, and 20% methanol for 15 min, with two changes in buffer solution. The proteins in gel were transferred to a 0.45-μm nitrocellulose filter at 1.2 ml/cm² for 40 min. The nitrocellulose filter was immersed in 15 ml of block ace at 4 °C overnight for blocking. The filter was then dipped into 15 ml of buffer B consisting of 0.05% Tween 20, 5% block ace in PBS, and the antibody of interest in 15 ml of buffer C consisting of 0.01% bromophenol blue. The filter was washed three times with buffer B and then immersed in 15 ml of buffer B containing peroxidase-fixed goat anti-rabbit IgG (1 μg/ml) for 1 h at room temperature and then washed five times with buffer B. The antigen, Epo-R, was visualized using the enhanced chemiluminescence Western blotting detection system.

**Nucleotide Sequence of Epo-R cDNA from PC12 Cells and Rat Erythroid Cells**—The coding regions of Epo-R cDNAs from anemic rat spleen cells and PC12 cells were obtained using the RT-PCR method (25). Primers N (5'-GGCAAGCTTGCTGCTGCAACCTGCG-3'), C (5'-GGCTTAGATGAGCCTGAAGTCCTAC-3'), F (5'-GCTGTCG-AGCCACCTGCGG-5'), and R (5'-GTCGAGATGCTATCATA-3') were synthesized to amplify the coding region of Epo-R from PC12 cells and rat erythroid cells. The underlined sequences in the primers N, C, F, and R correspond to the mouse Epo-R cDNA sequences, 18-30, 1564-1549, 640-656, and 1314-1296, respectively (numbered according to Ref. 26). RT-PCR using primers N and C amplifies the whole coding region of Epo-R. The 5' eight nucleotides in primers N and C were added for creation of restriction sites. Single-stranded cDNAs were synthesized by Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) using poly(A)⁺ RNA from anemic rat spleen cells and PC12 cells as templates. PCR was performed between primers N and C, N and R, or F and C using single-stranded cDNAs as templates. Nucleotide sequences of RT-PCR products were determined by the direct sequencing method (27) of cloned RT-PCR products by the dye-exchange method using Sequences (28).
determined using the fluorescent calcium indicator fura-2 (29, 30). PC12 cells were loaded with 10 μM fura-2-AM, the ester form of fura-2, by incubating the cell suspensions in PBS at 2 × 10⁶ cells/ml for 45 min at 37 °C. The cells were washed three times by an isotonic buffer consisting of 125 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 5 mM NaHCO₃, 1 mM CaCl₂, 6 mM glucose, 25 mM HEPES, and 1 mM CaCl₂ and resuspended in the same buffer. One-milliliter aliquots of the cell suspension were pipetted into tubes and the tubes were kept on ice for 1 h. Cells were resuspended in the same buffer warmed at 37 °C. Fluorescence measurements were made in 1-ml samples continuously stirred in quartz-glass cuvettes and thermally maintained at 37 °C. Fluorescence was monitored with a Shimadzu RF-5000 spectrophotometer, with excitation at dual wavelengths (340 and 380 nm) and emission at 490 nm; ratios of fluorescence intensities emitted when excited at 340 and 380 nm were recorded.

Intracellular Monoamine Concentrations of PC 12 Cells—PC12 cells (1.2 × 10⁶ cells/3 ml of medium) were cultured in a 35-mm plastic dish for 2 days in the presence of Epo at 3 nM or in its absence. Cells were thoroughly washed with PBS and suspended in 100 μl PBS. An aliquot (20 μl) of the suspension was used for measurement of protein according to Lowry's method after the lysis of cells in 1% SDS. The remainder (80 μl) of the cell suspension was homogenized with 200 μl of 0.1 M perchloric acid. After centrifugation, the supernatant was precipitated almost completely by 5% trichloroacetic acid and therefore the radioactivity was determined using the fluorescent calcium indicator fura-2 (29, 30). PC12 cells were loaded with 10 μM fura-2-AM, the ester form of fura-2, by incubating the cell suspensions in PBS at 2 × 10⁶ cells/ml for 45 min at 37 °C. The cells were washed three times by an isotonic buffer consisting of 125 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 5 mM NaHCO₃, 1 mM CaCl₂, 6 mM glucose, 25 mM HEPES, and 1 mM CaCl₂ and resuspended in the same buffer. One-milliliter aliquots of the cell suspension were pipetted into tubes and the tubes were kept on ice for 1 h. Cells were resuspended in the same buffer warmed at 37 °C. Fluorescence measurements were made in 1-ml samples continuously stirred in quartz-glass cuvettes and thermally maintained at 37 °C. Fluorescence was monitored with a Shimadzu RF-5000 spectrophotometer, with excitation at dual wavelengths (340 and 380 nm) and emission at 490 nm; ratios of fluorescence intensities emitted when excited at 340 and 380 nm were recorded.

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RESULTS

Binding of Epo to Rat Neuronal and Erythroid Cells—Fig. 1A shows the time-dependent binding of ¹²⁵I-rHuEpo to PC12 cells. The maximum specific binding occurred within 3 h of incubation at 15 °C. There were proportional increases in the specific binding as the number of PC12 cells increased (Fig. 1A). Similar results for the time- and cell number-dependent binding were obtained with SN6 cells and rat spleen cells enriched with Epo-responsive cells (data not shown). Scatchard transformation of ligand-saturation curves of PC12 and SN6 cells yielded straight lines (Fig. 1C), indicating that these cells had a single class of binding sites. There were 1390 sites/cell with a Kd (dissociation constant) of 16 nM on PC12 cells and 84 sites/cell with a Kd of 10 nM on SN6 cells. A Scatchard plot of rat spleen erythroid cells was biphasic (Fig. 1D); erythroid cells had high-affinity binding sites (33 sites/cell, Kd = 95 pm) and low-affinity binding sites (252 sites/cell, Kd = 1.9 nM). We examined whether the binding of Epo to PC12 cells was reversible. Radioiodinated rHuEpo was bound to cells by incubating the binding mixture at 15 °C for 3 h. The cells were washed thoroughly to remove the free ligand. The washed cells were again incubated at 15 °C, and the decrease in the bound ligand during incubation was measured. Fig. 2 shows the time-dependent release of radioactivity from PC12 cells and rat erythroid cells. The dissociated radioactivity was precipitated almost completely by 5% trichloroacetic acid and migrated in SDS-polyacrylamide gel with a molecular size similar to that of ¹²⁵I-rHuEpo; binding of Epo to PC12 cells as well as to erythroid cells is reversible. Epo appears to dissociate more rapidly from PC12 cells than erythroid cells. Since the unlabelled Epo is not added in the ligand-dissociation mixture, however, the time-dependent release of Epo in Fig. 2 reflects both dissociation of Epo from the cells and reassociation of the dissociated ligand.

Table 1 shows that the number of PC12 cells that were internalized at 37 °C but not at 15 °C. After the cells were incubated with ¹²⁵I-rHuEpo at 15 or 37 °C, the cell-associated Epo was exposed to a neutral pH or an acidic pH and then centrifuged. At the acidic pH the ligand bound to the cell surface receptor would be released from the cells, and therefore the radioactivity should appear in the supernatant after centrifugation. But the internalized ligand would not be released from the cells upon low-pH treatment, and therefore, the radioactivity...
should be associated with the pelleted cells. When Epo was bound to the cells at 15 °C and then the cell-associated Epo was exposed to the neutral pH, most of the radioactivity was associated with the pelleted cells. The radioactivity, however, was almost completely removed from the cells when the cell-associated Epo was exposed at the acidic pH. When the binding was done at 37 °C, approximately 40% of the total specific binding was still cell-associated after exposure to the acidic pH, in agreement with erythroid cells (21). The specific binding of Epo to PC12 cells at 15 °C is indeed equivalent to the receptor-bound ligands on the cell surface, but a significant portion of the ligands bound to the cells at 37 °C is internalized.

The culture of PC12 cells in the presence of NGF caused neurite growth. When Epo binding was tested for PC12 cells cultured for 3 days in the presence of 2 nM NGF, there were no significant changes in the characteristics of Epo binding, including the number of binding sites.

PC12 cells were derived from rat adrenal chromaffin cells (6). The natural chromaffin cells were prepared from rat adrenal medullas, and the specific binding of 125I-rHuEpo at 10 nM was tested using 4 x 10^6 cells. There was specific and reproducible binding (410 ± 20 cpm in triplicate assays), but low numbers of the available cells did not allow us to carry out further characterization of the binding.

Specificity of Binding of Epo to PC12 Cells—Affinities of Epo binding sites on PC12 and SN6 cells are much lower than those on erythroid cells, and Epo is a heavily glycosylated protein (1, 16). It is possible that the Epo binding to these neuronal cells occurs through recognition of sugar chains attached to Epo, like a lectin-glycoprotein interaction. This possibility, however, was excluded by the results that the deglycosylated form of rHuEpo inhibited the specific binding of 125I-rHuEpo with a potency similar to that of the fully glycosylated rHuEpo and that metal chelators (EGTA and EDTA) and monosaccharides (GalNAc and Gal), which are inhibitors of the interaction among some lectins and glycoproteins, showed no effect on the specific binding (Table II). Rat Epo also inhibited the specific binding of 125I-rHuEpo with an efficiency similar to that of the unlabeled rHuEpo, indicating that the low affinity of PC12 cells was not due to a combination heterogeneous in origin of the ligand and target cells. The monoclonal antibodies (R2 and R6) against rHuEpo decreased the binding, probably by deprivation of free ligand, but the control antibody directed against transglutaminase

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**Table I**

**Internalization of Epo bound to PC12 cells**

Radiiodinated rHuEpo was bound to PC12 cells under the different conditions, and the cells were then separated from the free ligand as described under "Experimental Procedures." The cells were subjected to the postbinding treatment for 3 min on ice and centrifuged. Radioactivity of the supernatant and pelleted fractions was counted. Radioactivity due to nonspecific binding was measured with the experiments in which 200-fold unlabeled Epo was added to the binding mixtures. Each value is the mean of duplicate assays ± deviation of duplicate values. The values in parentheses indicate specific binding. Total radioactivity represents the sum of supernatant and pellet radioactivity.

| Temperature and time | Unlabeled Epo | Postbinding treatment | Radioactivity cpm/10^6 cells |
|----------------------|---------------|-----------------------|-----------------------------|
|                      |               |                       | Supernatant | Pellet | Total  |
| 15 °C, 3 h           | -             | pH 7.4                | 194 ± 14   | 3064 ± 87 | 3258 ± 73 |
|                      | +             |                       | 134 ± 7    | 1528 ± 56 | 1662 ± 49 |
|                      |               |                       | (67)       | 1536   | 1596   |
|                      | -             | pH 2.5                | 2850 ± 44  | 472 ± 30   | 3322 ± 15 |
|                      | +             |                       | 1356 ± 63  | 365 ± 45   | 1719 ± 108 |
|                      |               |                       | (1494)     | 109      | 1603    |
| 37 °C, 1 h           | -             | pH 7.4                | 235 ± 49   | 3738 ± 6   | 3973 ± 43 |
|                      | +             |                       | 121 ± 14   | 1359 ± 81  | 1460 ± 95 |
|                      |               |                       | (114)      | 2399     | 2513    |
|                      | -             | pH 2.5                | 2563 ± 102 | 1369 ± 28  | 3932 ± 130 |
|                      | +             |                       | 1081 ± 44  | 361 ± 32   | 1442 ± 12  |
|                      |               |                       | (1482)     | 1008     | 2490    |
TABLE II
Specific binding of Epo to PC12 cells

| Substances                  | Concentration | Specific binding of \textsuperscript{125}I-rHuEpo |
|-----------------------------|---------------|-----------------------------------------------|
| rHuEpo                      | 5 nM          | 2592 ± 46                                     |
| Deglycosylated rHuEpo       | 5 nM          | 1470 ± 18                                     |
| Rat Epo                     | 5 nM          | 1486 ± 19                                     |
| GalNAc                      | 10 mM         | 2545 ± 76                                     |
| Gal                         | 5 mM          | 2348 ± 49                                     |
| EGTA                        | 10 mM         | 2768 ± 103                                    |
| EDTA                        | 15 mM         | 2027 ± 33                                     |
| R2*                         | 40 μg/ml      | 1083 ± 30                                     |
| R6*                         | 80 μg/ml      | 206 ± 24                                      |
| Anti-TG*                    | 40 μg/ml      | 2454 ± 67                                     |

\* rHuEpo-directed monoclonal antibodies (15).
\textsuperscript{a} Transglutaminase-directed monoclonal antibodies (41).

Specific binding was calculated from the mean value.

shown little effect. No inhibition of the binding was found in the presence of the following individual growth factor at 20 nM IL-1β, 20 nM IL-3, 200 nM IL-6, 200 nM G-CSF, 200 nM GM-CSF, 200 nM EDF (activin A), 200 nM tumor necrosis factor, 80 nM hepatocyte growth factor, 1 μM epidermal growth factor, 200 nM NGF, and 20 μM insulin. These properties of the Epo binding sites on PC12 cells demonstrate that the cells express Epo-specific receptors which interact with the polypeptide part of the ligand.

Cross-linked Products—Epo receptors on PC12 cells and rat erythroid cells were affinity-labeled by a chemical cross-linker, solubilized with Triton X-100, and then analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions (Fig. 3). There were two cross-linked products of rat erythroid cells with the molecular masses of 120 and 140 kDa, and a single product of PC12 cells with a 105-kDa molecular mass. These products were not detected when an excess of unlabeled Epo was added to the binding mixture. Subtracting the molecular mass of Epo, 35 kDa, from those of the cross-linked products gave the apparent size of the component cross-linked to Epo in each product; 85 and 105 kDa of erythroid cells and 70 kDa of PC12 cells.

Immunoochemical Detection of Epo-R of PC12 Cells and Erythroid Cells—Rabbit anti-mouse Epo-R antiserum was prepared by using the soluble form of mouse Epo-R as an antigen. This antiserum was used to identify Epo-R of erythroid cells and PC12 cells. Epo-R was solubilized from cells and concentrated by the Epo-fixed gel. The concentrated Epo-R was detected by the Western blotting technique (Fig. 4). Epo-R of mouse erythroid cell line, TSA8, was detected as a single band with a molecular size of 68 kDa (lane 1). A band with a similar size was found for rat spleen cells (lane 2). These bands were undetectable when the antiserum was preadsorbed with the antigen, soluble form of mouse Epo-R (lanes 4 and 5). PC12 cells yielded one major band at 62 kDa, and two additional minor bands at 58 and 54 kDa (lane 3). The major 62-kDa band appears to be Epo-R on PC12 cells, because the minor bands were still detected by the preadsorbed antiserum (lane 6). Experiments using the anti-NH\textsubscript{2}-terminal antiserum also showed Epo-R of 68 kDa in TSA8 and 62 kDa in PC12 cells.

Northern Blotting of Epo-R mRNA—Poly(A)\textsuperscript{+} RNA was isolated from fetal mouse liver cells, rat spleen cells, and PC12 cells and subjected to Northern hybridization using rat Epo-R cDNA as a probe (Fig. 5). The rat mRNA hybridizes with mRNA from fetal mouse liver cells (lane 1), rat spleen cells (lane 2), and PC12 cells (lane 3). The size (2.1 kilobases) of rat mRNA agrees with that from mouse cells (26, 31).

Nucleotide Sequence of Epo-R of PC12 Cells and Rat Erythroid Cells—The affinity of Epo to PC12 Epo-R is very low as compared with that of erythroid cells (Fig. 1), and the size of PC12 Epo-R is smaller than that of erythroid cells (Fig. 4). In order to know whether this low affinity is due to expression of a deletion-mutant Epo-R on PC12 cells, we determined the nucleotide sequence of the entire coding region of Epo-R cDNA from rat erythroid cells and PC12 cells. The nucleotide sequence of PC12 cells was identical to that of rat erythroid cells. Fig. 6 shows the nucleotide sequence of rat Epo-R and its deduced amino acid sequence; the mature rat Epo-R consists of 483 amino acids, and its calculated molecular weight is 52,794. For comparison, the amino acid sequences of mouse (26) and human (32, 33) Epo-R are shown. The amino acid sequences of the matured proteins were 82% conserved between the rat and human Epo-Rs. Homology between rat and mouse increased to 94%. Insertion of one amino acid occurs at position 49 in human Epo-R. The
presence of 4 spaced cysteines near the amino terminus (positions 28, 38, 66, and 82) and a WS motif to which receptors of Epo, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, transmembrane domain. This region is homologous to the IL-cDNA probe.

One putative N-glycosylation site (NYS at positions 51-53) is conserved in the carboxyl terminus have been proposed as a negative tyrosine kinase activity (38-40). Forty amino acid residues in a highly conserved region (positions 418-446) whose significance of Epo-R in nerve cells is to demonstrate production of Epo in brain tissue.

Immunochemical detection of solubilized Epo-R showed that the size (62 kDa) of Epo-R from PC12 cells was smaller than that (68 kDa) from rat erythroid cells. Analyses of PC12 mRNA by Northern blotting and its nucleotide sequence, however, were indicative of expressing neither a mutated Epo-R nor an alternative splicing-derived Epo-R. Neural cells may differ from erythroid cells in post-translational processing of Epo-R, resulting in expression of Epo-R with different sizes. One putative N-glycosylation site existing in the extracellular domain of Epo-R could be a cause for the size difference, but the difference could also result from other processing such as proteolysis and phosphorylation. Proteolytic removal of the NH2-terminal region from PC12 Epo-R is unlikely, because the antiserum against the NH2-terminal peptide reacts with the solubilized Epo-R.

The ligand affinity of Epo-R on neural cells (Kd = 10 ± 16 nM) is significantly lower than those on erythroid cells (Kd = 95 pm for high affinity site and 1.9 nM for low affinity site). The low affinity of Epo-R on PC12 cells might be related to a post-translational processing that yields Epo-R with a smaller size. But the N-linked sugar, if it causes the difference in Epo-R size, is not responsible for the affinity difference, because the N-glycosylation site-defective mutant of mouse Epo-R (44) and its extracellular soluble domain (22) are similar to the respective wild-type counterpart in binding with Epo.

A more intriguing hypothesis to account for the affinity difference is that there are accessory proteins that interact with Epo-R, altering interaction of Epo-R with the ligand. Cross-linking experiments revealed the presence of two proteins with 105 and 85 kDa in erythroid cells and a 70-kDa protein in PC12 cells (the size of Epo, 35 kDa, has been subtracted from the cross-linked products) (see Fig. 3). The two proteins found in rat erythroid cells are consistent with enzyme in the biosynthetic pathway of the four monoamines that increased, is activated or accumulated.
The nucleotide sequence of rat Epo-R is presented in the first line. Amino acid sequences of rat (RT), mouse (MS), and human (HU) Epo-R are presented in the second, third, and fourth lines, respectively. Position 1 was given to the putative NH₂-terminal amino acid of the mature protein (26). The transmembrane domain is underlined.

Fig. 7. A Epo-induced increase in cytosolic calcium concentration of PC12 cells. A, Epo at 3 nM and bradykinin at 0.1 nM were added to the fura-2 AM-loaded PC12 cells at the indicated time. The ratio F340/F380 represents the ratio of fluorescence at 490 nm when excited at 340 nm to that excited at 380 nm. B, experimental conditions were as in A, except that 1 mM EGTA was present in the assay mixture. EGTA inhibits an Epo-induced increase in the cytosolic calcium concentration but does not inhibit a bradykinin-induced increase.
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FIG. 8. Effects of Epo on intracellular monoamine concentrations of PC12 cells. The control cells cultured without Epo contained DOPA, DOPAC, dopamine, HVA, and MDOPA with averages of 5.4, 2.2, 5.3, 0.5, and 0.7 ng/mg protein, respectively. These values were defined as 100%. Triplicate cultures were done with and without Epo. The values are means ± S.D.

those reported previously in rat source (45) and the presence of two components with similar sizes has been shown in erythroblasts of human and mouse (for review see Refs. 1 and 46). No conclusive evidence for these proteins being derived from cloned Epo-R cDNA has been reported. The molecular weight of Epo-R predicted from cloned mouse (26) and rat cDNA (this paper) is about 53,000, and the size of Epo-R detected by immunoblotting (Ref. 47 and this paper) or by ligand blotting technique (48, 49) is around 65 kDa; this size of Epo-R is much smaller than two proteins included in cross-linked products of erythroid cells. From these, it has been suspected that erythroid cross-linked products may consist of Epo, Epo-R, and an unidentified accessory protein. It has been reported recently, however, that erythroid cross-linked products before denaturation are precipitated by the antiserum against the cytoplasmic domain of mouse Epo-R, but, surprisingly, they are not precipitated upon denaturation (boiling of the cross-linked products for 5 min in Laemmli SDS-electrophoresis buffer) (50). The cross-linked products either before or after denaturation could be precipitated by the antiserum against Epo. These results have been interpreted as a strong indication of the following. 1) Epo interacts with those already reported (50). The 105-kDa cross-linked product before denaturation are precipitated by the antiserum against Epo. These results have been interpreted as a strong indication of the following. 1) Epo interacts with those already reported (50).

2) Epo-R is retained in complexes through noncovalent interaction with Epo and Epo-R; 2) Epo-R is cross-linked with accessory protein(s) which is immunochemically unrelated to Epo-R, and 3) before denaturation of cross-linked products, Epo-R is retained in complexes through noncovalent interaction with Epo or both Epo and an accessory protein, but Epo-R is dissociated from the complexes upon denaturation. By using the antiserum against the extracellular domain of mouse Epo-R, we performed similar experiments of erythroid cross-linked products, and the data obtained were consistent with those already reported (50). The 105-kDa cross-linked product of PC12 cells could not be precipitated either before or after denaturation, although our antiserum reacted with the solubilized Epo-R of PC12 cells on the Western blotting filter (see Fig. 4). The PC12-derived cross-linked product was precipitated by the antiserum against Epo, regardless of denaturation of the product. From these results of the PC12 cells, we infer that most of the Epo-R associated with the PC12 cross-linked product may have dissociated during solubilization of the cross-linked product so that the cross-linked product even before being subjected to the denaturation treat-

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