Characterization of the Structure of the Erythropoietin Receptor by Ligand Blotting

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Erythropoietin (Epo) regulates the growth and differentiation of erythroid cells by binding to a specific receptor. We characterized the native Epo receptor on erythroleukemia cell lines by ligand blotting. Solubilized cell membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose, and probed with 125I-Epo. Specificity was demonstrated by inhibition of 125I-Epo binding by unlabeled excess Epo but not other peptide growth factors and by the cellular distribution of the Epo binding protein. A single membrane protein of 81 Kd ± 4 Kd was sufficient to bind 125I Epo in both human (OCIM2, K562) and murine (GM979, Rauscher, DA-1) cell lines. This finding is consistent with the predicted size of the Epo receptor from the murine cDNA clone. However, chemical crosslinking of 125I-Epo to its receptor has identified two Epo binding proteins of 105 Kd and 85 Kd. This difference may occur because the receptor is size fractionated before Epo binding in the ligand blot, but after Epo binding in crosslinking studies. Ligand blotting demonstrates that the native Epo receptor is composed of a single 61-Kd Epo binding protein, and suggests the presence of additional proteins of 20 to 25 Kd that associate with the receptor after Epo binding.

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Materials and Methods

Materials. Radiolabeled recombinant human Epo was obtained from Amersham Corp (Arlington Heights, IL). Unlabeled purified human recombinant Epo (129,000 U/mg) and granulocyte-colony-stimulating factor (G-CSF) used for competitive binding studies were obtained from AMGEN (Thousand Oaks, CA). Epo for cell culture was partially purified (6,000 U/mg) from supernatant of BHK cells transfected with the Epo gene. Neutralizing anti-Epo polyclonal antibodies and preimmune serum were obtained from Dr Joan Egie of AMGEN. Acrylamide, N,N'-methylene-bis-acrylamide, bromophenol blue, SDS, TEMED, ammonium persulfate, glycine, and Tris(hydroxymethyl)aminomethane were obtained from BioRad (Richmond, CA). Prestained protein molecular weight markers and 14C-protein molecular weight markers were procured from Bethesda Research Laboratories Life Technologies, Inc (Gaithersburg, MD). Nitrocellulose filters were bought from Schleicher and Schuell Inc (Keene, NH). Reagent grade methanol was purchased from J.T. Baker Chemical Co (Phillipsburg, NJ). The protease inhibitors, aprotinin and leupeptin, and the detergent Triton X-100 were from Boehringer Mannheim Biochemicals (Indianapolis, IN), while phenylmethylsulfonyl fluoride (PMSF), benzanidine, and the remaining chemicals were obtained from Sigma Chemical Co (St Louis, MO). XRP autoradiography film was purchased from Eastman Kodak (Rochester, NY).

Cells. Rauscher Red 5, K562, U937, and U937 cell lines were cultured in RPMI 1640 (GIBCO Lab, Grand Island, NY) with 5% heat-inactivated calf serum (Hyclone Labs, Logan, UT). GM979, originally identified as T-Cl-2, 125I OCIM2, and HL60 were maintained in RPMI 1640 with 10% calf serum. DA-1 was cultured in RPMI 1640 with 5% calf serum and 2 U/ml of recombinant human Epo.

Cell fractionation. Enrichment for Epo receptors was accomplished by fractionating cells to remove their nuclei and soluble cytoplasmic components, using a method adapted from Paganelli et al. Approximately 10^6 cells in log phase growth were washed in phosphate-buffered saline (PBS; pH 7.4), resuspended in 2 ml of 10 mM Tris-HCl, 1 mM MgCl2, and incubated for 10 minutes on ice. The cells were homogenized with 10 strokes in a Dounce homogenizer at 4°C. Eight milliliters of ice-cold 250 mM sucrose was added to the homogenate. Cytocentrifuge preparation of the homogenate demonstrated greater than 90% lysis of the cells. The nuclear fraction and the residual undisrupted cells were pelleted by centrifugation at 300g for 5 minutes. The supernatant was centrifuged at 25,000g for 30 minutes at 4°C to pellet the plasma membranes and microsomes. The pellet was resuspended in 200 μl of 1% Triton X-100, 10 mM EDTA, 1

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mmol/L PMSF, 100 TIU/mL Aprotinin, 50 μg/mL Leupeptin, 25 mmol/L Benzamidine. The suspension was incubated on ice at 4°C for 15 minutes before removing the insoluble debris by centrifugation at 15,000 g for 15 minutes at 4°C. The extracted cell membrane proteins were stored at −20°C. This preparation retained binding activity on the ligand blot for at least 2 months under these storage conditions. For the dot blot analysis of growth factor competition, the solution was centrifuged at Triton X-100 in 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl. The solution was centrifuged at 100,000 g for 1 hour. The supernatant was stored at −70°C until use.

Ligand blot. Ligand blotting was performed as described by Bird et al. Fifty microliters of the detergent extract of fractionated cellular membrane was mixed but not boiled with 10 μL of 5X concentrated Laemmli SDS-PAGE sample buffer, without reducing agents, to yield solutions containing 125 mmol/L Tris-HCl pH 6.8/7.5% (vol/vol) glycerol/2% SDS/0.002% bromphenol blue. Electrophoresis of the sample was performed on 7.5% or 12.5% SDS-PAGE at 30 V for 18 hours at 4°C. The separated proteins were transferred to 0.22-μm nitrocellulose filters by electroblotting with 60 mA for 22 hours at 4°C in 25 mmol/L Tris (pH 8.3), 192 mmol/L Glycine, 20% (vol/vol) methanol buffer. The nitrocellulose filter was blocked for 24 hours in two 250 mL changes of blocking buffer (1% Carnation non-fat milk powder, 2% heat-inactivated bovine or equine serum in 10 mmol/L Tris-HCl [pH 7.4], 140 mmol/L NaCl, 0.005% Antifoam A). The nitrocellulose filter was incubated in 3 mL of blocking buffer containing 125I-Epo (3,000 Ci/mmol, final concentration of 100 to 400 pmol/L) with or without greater than 100-fold excess of unlabeled purified recombinant human Epo for 2 hours at 22°C. The filter was washed in two 250 mL changes of fresh blocking buffer and air dried. The Epo binding proteins were visualized by autoradiography at −70°C with DuPont Cronex Lightning Plus intensifying screens, generally for 3 to 5 days.

Dot ligand blot. Aliquots of Triton X-100 solubilized fractionated cellular membrane proteins were vacuum filtered onto 1-cm² pieces of 0.45-μm nitrocellulose filters using a Buchner funnel or a dot blotting apparatus. The filters were washed six to eight times with 10 mL of TBS (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl), then blocked, probed, and washed as outlined under ligand blotting. 125I-Epo binding was visualized by autoradiography. Quantitative analysis of 125I-Epo binding was performed by counting the nitrocellulose filter in a Packard 5330 gamma counter with a 66% counting efficiency.

RESULTS

Structure of the Epo receptor. Ligand blot analysis of 125I-Epo binding to GM979 cells showed a single Epo binding protein (Fig 1). In five experiments using GM979 cells, the molecular weight of this protein was estimated to be 61 ± 3 Kd (mean ± standard deviation) with a range of 55 to 64 Kd. In nine experiments using Rauscher cells, the molecular weight of this protein was estimated to be 61 ± 4 Kd (mean ± standard deviation) with a range of 53 to 64 Kd. No binding proteins were detected in the region between 14 Kd to 55 Kd when the solubilized cellular membrane proteins were size-fractionated on 12.5% SDS-PAGE. In one experiment, in which the major binding protein was visualized after 3 days of autoradiography, prolonged overexposure (24 days) of the ligand blot showed faint binding of 125I-Epo to a protein at 97 Kd. This band was not seen on any of eight subsequent trials in which additional protease inhibitors were used during the cell fractionation procedure, even with prolonged autoradiographic exposure time.

Coincubation of the ligand dot blot with excess unlabeled Epo resulted in a 79% (mean of five measurements) inhibition of 125I-Epo binding. 125I-Epo binding to the ligand blot was not inhibited by coinucubation with a 100-fold molar excess of either insulin or G-CSF (Fig 2). 125I-Epo binding to the ligand blot was inhibited by incubation with neutralizing anti-Epo polyclonal antibodies, but not by incubation with rabbit preimmune serum (data not shown). When increasing amounts of fractionated Rauscher cell membrane...
extract were applied to the ligand blot, an increased amount of $^{125}$I-Epo was bound (Fig 3). Increasing concentrations of $^{125}$I-Epo in the incubation mixture increased the amount of $^{125}$I-Epo bound to the ligand blot (Fig 4). Boiling the cellular membrane samples or treating the proteins with 2-mercaptoethanol before SDS-PAGE completely abrogated $^{125}$I-Epo binding (data not shown).

**Comparison of human and murine cell lines.** Both murine erythroleukemia (MEL) cell lines (GM979, Rauscher, and DA-1) and human erythroleukemia cell lines (OCIM2 and K562) specifically bound $^{125}$I-Epo in the ligand blot (Fig 5). These cells have been previously shown to express between 30 and 2,000 Epo receptors per cell (V. Broudy, unpublished data). Two human non-erythroid cell lines that do not express Epo receptors (U937 and HL-60) did not bind $^{125}$I-Epo in the ligand blot (Fig 5). The molecular weight of the Epo binding protein was similar in murine and human cells, as well as responsive (DA-1) and nonresponsive cell lines (GM979, Rauscher, K562, OCIM2).

**DISCUSSION**

In the present study, we used ligand blotting to demonstrate the presence of a single 61-Kd Epo binding protein in solubilized proteins from erythroleukemia cell lines. Other investigators have developed a dot assay for the Epo receptor but have not used this approach to determine the size of the receptor. We found that expression of the Epo binding protein was restricted to Epo receptor-bearing cell lines. The binding of $^{125}$I-Epo to the 61-Kd protein was competitively inhibited by excess unlabeled Epo and by neutralizing anti-Epo polyclonal antibodies, but not by other peptide growth hormones. The use of an increasing concentration of $^{125}$I-Epo as a probe, or the application of greater amounts of membrane proteins enhanced the quantity of $^{125}$I-Epo bound to the ligand blot. These data demonstrate the specific nature of $^{125}$I-Epo binding in the ligand blot and show that the Epo binding polypeptide of the native Epo receptor is a 61-Kd protein. The molecular weight of the native Epo receptor identified by ligand blotting agrees with the predicted molecular weight of the protein encoded by the murine and human Epo receptor cDNAs and with the size of the protein expressed from the murine Epo receptor DNA detected by either immunoprecipitation or precipitation with biotinylated Epo.

In contrast to the results obtained by ligand blotting, crosslinking of $^{125}$I-Epo to its receptor generally demonstrates two complexes, corresponding to estimated molecular weights of 100 and 85 Kd for the receptor proteins after accounting for the size of Epo in the complex. These complexes have been seen on normal murine, rat and human erythroid precursors, murine and human cell lines, and placental tissue. In some reports, electrophoresis of the crosslinked Epo–Epo–receptor complex under nondenaturing conditions demonstrates a single higher molecular weight species of 224 Kd that can be separated into a 136-Kd
variable glycosylation, the existence of two distinct Epo binding protein was seen in one trial after prolonged the receptor. Although a faint higher molecular weight molecular weight protein than that observed with Iz5I-Epo binding protein did not change when greater numbers and 119-Kd components on red~cti0n.l~ The proteins com-

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Isolation of the 1251-Epo receptor complex in a mixture of 11 protease inhibitors did not abolish the presence of the 85-Kd Epo receptor complex.45 Thus, the observation of two crosslinked Epo receptor complexes is not explained by variable glycosylation, the existence of two distinct Epo receptor proteins, or partial proteolysis.

It is unlikely that ligand blotting identifies a lower molecular weight protein than that observed with 125I-Epo affinity crosslinking because of proteolytic degradation of the receptor. Although a faint higher molecular weight binding protein was seen in one trial after prolonged autoradiographic exposure, in eight other experiments a single sharp band of 125I-Epo binding was seen at 61 Kd. Proteolytic enzyme inhibitors were included at every step of the sample preparation. The molecular weight of the Epo binding protein did not change when greater numbers and higher concentrations of proteolytic inhibitors were used.

The differences in size and number of receptor proteins identified by ligand blotting and affinity crosslinking could be due to labeling of Epo receptor proteins by affinity crosslinking that do not bind 125I-Epo on the ligand blot. One would have to postulate that the 61-Kd Epo receptor identified by ligand blotting, a receptor that demonstrates specific ligand binding, a correct cellular distribution, and that agrees with the size of the receptor predicted by the cDNA for the Epo receptor, is not recognized when 125I-Epo is crosslinked to intact cells. The number of conditions involved in this model make it unlikely.

Epo binding on the ligand blot, measured by the intensity of autoradiographic bands, may not correlate to reported values for the number of receptors per cell for several reasons. First, the binding affinity of Epo for its receptor varies between cell lines. The intensity of binding on the ligand blot reflects both binding affinity and receptor number. Second, studies quantitating binding of 125I-Epo to intact cells are usually performed under conditions that minimize internalization of Epo, thus providing a measure of cell surface receptors. Our ligand blot technique identifies all membrane-associated Epo receptors in the cell.46 Third, recovery of Epo receptors during membrane prepara-

tion may vary among the cell lines.

Although both crosslinking studies and ligand blotting depend on the specificity of the ligand-receptor interaction to identify a receptor, there are two major methodologic differences between these techniques. First, ligand blotting permits the analysis of receptor structure without using reagents that chemically modify either the receptor or its ligand. Second, in ligand blotting, the receptor is size fractionated before ligand binding. This procedure differs from crosslinking studies, in which the ligand is covalently attached to its receptor and the complex is then size fractionated. Thus, differences in size and subunit structure seen between crosslinking and ligand blotting may be due to an alteration in the Epo receptor caused by Epo binding.

We propose that the Epo binding protein is the 61-Kd protein identified by ligand blotting and predicted by the cDNA clone encoding the Epo receptor.26,29,36 Based on these data we hypothesize that, on Epo binding, there is a ligand-dependent association between the receptor and a 20- to 25-Kd Epo receptor-associated polypeptide in a 1:1 or 1:2 stoichiometry. The 20- to 25-Kd subunits do not bind Epo, as demonstrated by ligand blot experiments. This model would predict the presence of a single Epo binding protein before Epo binding, consistent with the ligand blotting experiments. It would also predict two distinct but similarly composed receptor complexes of 85 and 100 Kd postreceptor binding, consistent with the crosslinking and peptide mapping experiments.46 Heterodimeric multisub-unit receptor structures have been proposed for the interleukin-2 (IL-2) receptor37 and the IL-6 receptor,48 which are members of the same growth factor receptor family as the Epo receptor.49

Discrepancies between receptor size estimates determined by affinity crosslinking and either ligand blotting or cloning of the receptor cDNA have been reported for other receptors. Chemical crosslinking of 17.5 Kd 125I-IL-1 to murine T-cell lines identified a major binding complex of 120 to 130 Kd and a minor binding complex of 95 to 100 Kd, suggesting the IL-1 binding proteins are 105 and 80 Kd.40 Ligand blotting of the same cell line identified a single 80-Kd binding protein for the IL-1 receptor that is identical in molecular weight to the protein encoded by the isolated murine T-cell IL-1 receptor cDNA.49 Complexes of 158 and 105 Kd are identified by affinity crosslinking of 125I nerve growth factor (NGF) to a rat pheochromocytoma cell line, suggesting that the NGF binding proteins are 143 and 87 Kd.31 However, the cDNA for the rat NGF receptor encodes an 83-Kd glycoprotein.32 Accessory NGF receptor-associated proteins may account for the larger crosslinked NGF receptor complex observed.32,53

Finally, the Epo-responsive murine cell line SKT6 demon-

strated three Epo binding proteins of 119, 94, and 63 Kd, while a nonresponsive murine erythroleukemia cell line had only one binding protein of 63 Kd.7 Todokoro et al raised the possibility that the association of the 63-Kd receptor protein with another set of proteins (resulting in a receptor complex of 119 and 94 Kd) is required for the functioning of the receptor.7 Other investigators have found the structure of the Epo receptor to be similar in Epo-responsive and nonresponsive cells.5,8,13,20,25 In our study, Epo-responsive and Epo-nonresponsive cell lines expressed an Epo receptor of the same molecular weight, suggesting that loss of responsiveness is not due to a gross alteration (such as the truncation of a large region of the signalling domain) of the receptor.

In summary, ligand blotting of the native Epo receptor demonstrates that the Epo binding protein is a single polypeptide of 61 Kd. The molecular weight of the receptor on both human and murine cell lines, and on responsive and nonresponsive cell lines, is similar. Finally, we propose that binding of Epo to its receptor may trigger association of additional polypeptides, resulting in a change in the structure of the receptor.
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