Subunit Structure of the Erythropoietin Receptor*

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Chemical cross-linking of the red blood cell hormone, erythropoietin (Epo), to its receptor on erythroid cells has revealed the presence of two proteins closely associated with Epo, but the relationship between these two proteins is controversial. Using the cross-linking reagents disuccinimidyl suberate and dithiobisuccinimidy1 propionate, we show that $^{125}$I-Epo can be specifically conjugated in a complex of 224 kDa using mouse fetal liver cells, bone marrow cells, and Friend virus-induced splenic erythroblasts as demonstrated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels under nonreducing conditions. Under reducing conditions, the 224-kDa complex appeared as two Epo conjugates of 136 kDa and 119 kDa, and these bands were also observed to a variable extent in some nonreducing gels. Disulfide linking of the 136-kDa and 119-kDa bands was confirmed by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis run under nonreducing followed by reducing conditions.

With increasing time of $^{125}$I-Epo binding to Friend virus erythroblasts in the presence of sodium azide to inhibit receptor internalization, the 136-kDa and 119-kDa bands seen under reducing conditions increased markedly in intensity, whereas the 224-kDa band seen under nonreducing conditions declined. These results suggest that the 224-kDa Epo conjugate is inefficiently solubilized under nonreducing conditions following prolonged periods of Epo binding.

This suggests that high affinity receptors for Epo on each of the cell types tested is demonstrated. It is concluded that the two disulfide-linked Epo-binding proteins which can be independently cross-linked to Epo form a single ligand binding site.

**EXPERIMENTAL PROCEDURES**

Materials—Human recombinant Epo was obtained from Amgen Biologics (Thousand Oaks, CA) as a carrier-free product (104,000 units/mg) or as TC Epo (4000 units/ml) containing 0.025% bovine serum albumin (BSA). Na$^{125}$I was obtained from Amersham, and disuccinimidyl suberate (DSS) and dithiobisuccinimidy1 propionate (DSP) were obtained from Pierce. Friend virus (SFFV/FRE cl-3/MoLV(201)) was obtained from Dr. S. B. Krantz (Vanderbilt, TN) and was passaged in BALB/c mice hied and maintained at the Wellington School of Medicine. Molecular weight standards were from Boehringer Mannheim.

Cells—Fetal liver cells were obtained from timed pregnancies of CBA x C57BL/6 mice at 14–16 days. Livers were dispersed in phosphate-buffered saline, pH 7.4 (PBS) using a Pasteur pipette, allowed to settle at 1 °C for 5 min, and the suspended cells were recovered by centrifugation at 1000 × g for 5 min and washed in PBS. Bone marrow cells were flushed from the femurs and tibias of CBA (CBA x C57BL/6) mice of either sex, and a single cell suspension in PBS was prepared as described above for fetal liver. Fetal liver cell preparations contained 45% of nucleated cells as erythroblasts, whereas erythroid cells in bone marrow comprised 6–7% of nucleated cells.

Friend virus spleen cells were obtained from BALB/c or (BALB/c x DBA/2)F, mice that had been inoculated 15–20 days previously with 0.2 ml of a 1:20 dilution of fresh or frozen plasma from 2–5 week infected mice. Spleens were teased apart, the cells were dispersed through a stainless steel sieve, and a single cell suspension was prepared as with fetal liver. Cells were fractionated on Ficol/Metrizoate ($p = 1.09$ g/ml) at 800 × g for 20 min. and cells at the interface were washed twice with PBS. The resulting cell preparation contained 83–86% erythroblasts.

Iodination of Epo—Carrier-free human recombinant Epo (1–2 μg) was radioiodinated by the two-phase chloramine-T procedure of Tejedor and Ballesta (11), except that chloramine T was dissolved in 0.025 M borate buffer, pH 8.5. The filter was changed four times at 10-min intervals, and $^{125}$I-Epo was separated on a Bio-Gel P-6 (Bio-Rad) column equilibrated with PBS containing 0.02% Tween 20 (Sigma) and 0.05% BSA. Radiolabeled Epo was stored frozen at −70 °C and had a specific radioactivity of 4–10 × 10$^6$ cpm/μg (0.5 μg/μl) amounting to 0.6–1.2 iodine atoms incorporated per molecule of...
Epo. $^{125}$I-Epo retained full biological activity as determined in a fetal liver CFU-E assay (12).

**Epo Binding Assay**—Cells (2 x $10^5-1.5 x 10^5$) in 0.1-0.2 ml PBS, containing 0.1% BSA and 0.02% sodium azide to inhibit receptor internalization, were incubated in polypropylene tubes with (a) $^{125}$I-Epo and (b) $^{125}$I-Epo in the presence of 38-66-fold excess unlabeled TC Epo at 37 °C for 90 min with occasional mixing. Cell-associated Epo was separated from free Epo by centrifugation through a 0.4-ml cushion of 10% BSA in PBS at 4000 g for 5 min. The supernatant was aspirated, the pellet washed in PBS containing 0.1% BSA and 0.02% sodium azide, and radioactivity in the cell pellet measured in a y-counter. By using this procedure, less than 15% of $^{125}$I-Epo was internalized as determined by radioactivity retained following a pH 4 wash (13), and >80% reversibility was shown by reincubating cells at 37 °C for 90 min in the absence of Epo.

**Chemical Cross-linking**—Cross-linking was carried out essentially as described by Nicola and Peterson (14) by adding 2 μl of 50 mg/ml DSS or DSP dissolved in dimethyl sulfoxide to cells (0.1 ml in PBS) with bound $^{125}$I-Epo. The cell suspension was incubated at 6 °C for 40 min with occasional mixing, then centrifuged in a Beckman Microfuge B at 8740 x g for 10 s. The supernatant fluid was removed, and the cell pellet was stored frozen at −70 °C. Frozen cells were thawed at 20 °C, and 20 μl of 20 mM Tris/HCl, pH 7.4, was added. In some experiments, oxidized glutathione (2 μl of 100 mM, Sigma) was added at this stage to minimize the reducing environment generated during cell lysis. The cells were then frozen and thawed a further three times between liquid nitrogen and a water bath at 40 °C, MgCl₂ (6 μl of 0 mM) and DNase I (Sigma, 2 μl of 40 units/ml) were added, and the mixture was maintained on ice for 60 min. MgSO₄ (2 μl of 50 mM) was then added followed by 2 μl of a mixture of EDTA (0.1 M), aprotinin (Sigma, 5000 units/ml), and phenylmethylsulfonyl fluoride (Sigma, 10 mM). SDS-sample buffer (30 μl) containing 5% mercaptoethanol for reducing gels was added, and the mixture was heated at 90 °C for 5 min unless otherwise stated. The solubilized samples were centrifuged at 8740 x g for 10 s in a Beckman Microfuge, and the supernatant was electrophoresed on 7.5% or 5-15% gradient SDS-polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R-250, destained, and dried under vacuum onto Whatman No. 9MM filter paper. Dried gels were exposed to Kodak XRF x-ray film for 1-8 weeks using Cronex Lightning Plus calcium tungstate intensifying screens. Two-dimensional gels were run in the first dimension on 7.5% polyacrylamide tube gels (2.5-mm diameter) using a 1-cm stacking gel. This gel was reduced with 4 ml of 1.3-diluted sample buffer containing 10% 2-mercaptoethanol at 60 °C for 12 min and layered onto a 7.5% polyacrylamide slab gel using agarose containing 12% 2-mercaptoethanol. Molecular mass markers were from Boehringer Mannheim.

**RESULTS**

**Disulfide Linkage of Polypeptides Associated with $^{125}$I-Epo**—$^{125}$I-Epo was cross-linked to its receptor on fetal liver cells with the homobifunctional reagent DSS. Fig. 1A shows that, under reducing conditions, two major radiolabeled conjugates were observed having relative molecular masses of 136 kDa and 119 kDa on 7.5% SDS-polyacrylamide gels. These bands were not observed when binding was carried out in the presence of a 40-fold excess of unlabeled Epo (Fig. 1A, lane 1). Use of an alternative cross-linker, DSP having a cleavable disulfide bridge, produced a different pattern of bands. Fig. 1B shows that, under nonreducing conditions, Epo conjugates of 224 kDa, 90 kDa, and 74 kDa were evident. The absence of bands of 136 kDa and 119 kDa with DSP suggests that these two polypeptides may be disulfide-linked in a heterodimeric complex. To test this possibility, fetal liver cells with bound $^{125}$I-Epo were cross-linked with DSP, and the proteins were analyzed on 5-15% SDS-polyacrylamide gels using an intermediate nonreducing lane to generate a gradient between the nonreducing and reducing lanes. Fig. 2A shows that the 224-kDa band observed under nonreducing conditions (lane 1) was not seen under reducing conditions (lane 2) but appeared as a poorly resolved doublet of 136 kDa and 119 kDa. Radioactivity in the 110-129-kDa region of the nonreduced lane also appeared to contribute to the 136-kDa/119-kDa doublet following reduction (see intermediate "gradient," lane 2). Two smaller Epo conjugates of 90 kDa and 74 kDa, observed under nonreducing conditions, ran as a band of 76-82 kDa reduced. The possibility that the nonreduced bands of 110-129 kDa...
Subunit Structure of Erythropoietin Receptor

Non-reducing 3F5 5p4 934 170

Fig. 3. Analysis of 125I-Epo-associated polypeptides on mouse fetal liver cells by two-dimensional nonreducing/reducing SDS-polyacrylamide gel electrophoresis. 125I-Epo (2.7 nM) was bound to 15-day fetal liver cells (5 × 10^6) in absence (A) or presence (B) of a 60-fold molar excess of unlabeled Epo. Cells with bound Epo (4500 cpm specifically bound) or control cells were cross-linked with DSS, and cell lysates were electrophoresed on 7.5% SDS-polyacrylamide tube gels. Following treatment with 10% 2-mercaptoethanol, electrophoresis on 7.5% SDS-polyacrylamide slab gels was carried out in the second dimension. For comparison, a sample of cell lysate (1125 cpm of specifically bound 125I-Epo) was electrophoresed in the second dimension (Panel A, right lane). Material that did not enter the stacking and resolving gel in the first dimension is seen with vertical tailing. High molecular mass 125I-Epo-associated polypeptides falling below the diagonal of nondisulfide-bonded conjugates are shown with arrows.

FIG. 4. Comparison of affinity cross-linking of 125I-Epo to mouse fetal liver cells, bone marrow cells, and Friend virus-induced erythroblasts. 125I-Epo (2.7 nM) was bound to bone marrow cells (lanes 1–3), fetal liver cells (lanes 4–6), and Friend virus-induced erythroblasts (lanes 7–9) (0.21–1.7 × 10^6 cells in 0.15 ml). Cells with bound Epo (5–19 × 10^3 cpm per sample) were cross-linked with DSS, and oxidized glutathione was added to 10 mM prior to freeze-thawing. Cell lysates were analyzed on 5–15% SDS-polyacrylamide gels. Samples that were run on lanes 3, 4, and 9 were reduced with 5% 2-mercaptoethanol during cell solubilization.

may result from partial disulfide bond reduction of a larger conjugate prior to cross-linking, and electrophoresis was investigated by including the sulfhydryl blocking reagent iodoacetic acid during 125I-Epo binding and in all subsequent cell manipulations. Fig. 2B shows a similar pattern of bands in the presence of iodoacetic acid although the nonreduced bands were relatively less intense, and the 110–129-kDa band barely visible.

To confirm the apparent disulfide-linked structure of the 224-kDa conjugate, fetal liver cells with bound 125I-Epo were cross-linked with DSS, and the solubilized proteins were analyzed on two-dimensional nonreducing/reducing SDS-polyacrylamide gels. Fig. 3A shows that the nonreduced 224-kDa complex gave rise to two proteins of 136 kDa and 119 kDa (arrows) that dropped below the diagonal of non-disulfide-linked Epo-associated polypeptides. Radioactivity that

Fig. 5. Effect of time of binding, EGTA, and sodium azide on affinity cross-linking of 125I-Epo to Friend virus erythroblasts. 125I-Epo (2.7 nM) was bound to Friend virus-induced erythroblasts in the presence (lanes 1 and 10) and absence (lanes 2–9) of 36-fold excess unlabeled Epo for 20 min (lanes 5 and 6), 60 min (lanes 4 and 7), and 90 min (lanes 1–3 and 8–10). Cells with bound Epo were cross-linked with DSS and subjected to freeze-thawing and DNase treatment, and cell lysates were analyzed on 7.5% SDS-polyacrylamide gels under nonreducing (A) and reducing (B) conditions. EGTA (to 1 mM) was added 15 min prior to Epo binding in the samples shown in lanes 6–9, and sodium azide was omitted from samples analyzed in lanes 2 and 9.
subunit structure of the Epo receptor on fetal liver cells is compared with that on bone marrow and on Friend virus-induced erythroblasts in Fig. 4. The results suggest close similarity between Epo-associated proteins on each of these cell types, although an apparent increase in the mobility of the smaller Epo-conjugated bands was observed with Friend virus-induced erythroblasts in Fig. 4. The results suggest close similarity between Epo-associated proteins on each of these cell types, although an apparent increase in the mobility of the smaller Epo-conjugated bands was observed with Friend virus cells (Fig. 4, lanes 7–9).

Effect of Time of Binding on Proteins Conjugated to 125I-Epo—The experiments described above have used a 90-min incubation with 125I-Epo at 37°C in the presence of sodium azide to inhibit receptor internalization. The possibility that disulfide bridging may also be a feature of a small fraction of the Epo-conjugated proteins observed in this region.

The subunit structure of the Epo receptor on fetal liver cells is compared with that on bone marrow and on Friend virus-induced erythroblasts in Fig. 4. The results suggest close similarity between Epo-associated proteins on each of these cell types, although an apparent increase in the mobility of the smaller Epo-conjugated bands was observed with Friend virus cells (Fig. 4, lanes 7–9).

Fig. 5A shows that, at 20 min, a strong 224-kDa band was observed under nonreducing conditions (lane 5). As the time of incubation increased, the intensity of this band was markedly reduced (lanes 4 and 3). This change was also evident in the presence of 1 mM EGTA (lanes 6–8) and omitting azide from the binding mixture did not affect the 224-kDa species seen at 90 min (lanes 2 and 9). In contrast, under reducing conditions (Fig. 5B), the 136-kDa/119-kDa doublet was barely detectable following 20 min of incubation of cells with 125I-Epo (lane 5), but increased markedly at the 45-min and 90-min time points. This effect was also observed in the presence of EGTA (lanes 6–8). Omission of sodium azide reduced the appearance of the 136/119-kDa conjugates (lanes 2 and 9), and this effect was less evident in the presence of EGTA (lane 9) and consistent with receptor internalization. No consistent changes in the intensity of the lower molecular mass bands (<119 kDa) were observed between 20 and 90 min of Epo binding, either under nonreducing or reducing conditions. Furthermore, these bands were not consistently affected by EGTA or by omitting sodium azide from the binding mixture, suggesting that they may not be related to the functional Epo receptor. Except for a minor component at 70–80 kDa (Fig. 5, lanes 1 and 10), all radiolabeled bands were effectively competed out with unlabeled Epo.

Kinetics of 125I-Epo Binding to Fetal Liver, Bone Marrow, and Friend Virus Erythroblasts—To determine the kinetic association of Epo with its receptor on each cell type used in cross-linking studies, the binding properties of 125I-Epo were first established. With each cell type, binding was shown to be at equilibrium by 60–90 min, proportional to cell number, and reversible (results not shown). Binding studies were carried out at 37°C in the presence of 0.02% sodium azide to inhibit receptor internalization which was shown by pH 4 treatment to be less than 15% of specific bound radioactivity. Fig. 6 shows that with increasing concentrations of 125I-Epo, specific binding, as determined by the difference between total binding and binding in the presence of 40-fold excess unlabeled Epo, was saturable. Scatchard analysis (16) of three separate experiments with each cell type showed that the range of Kd was 414–488 pM for fetal liver cells, 675–705 pM for bone marrow cells, and 1.18–1.23 nM for Friend virus erythroblasts.

DISCUSSION

Chemical cross-linking has been widely used to characterize cell surface receptors for growth substances and hormones. We have used the homobifunctional reagents DSS and DSP to demonstrate that, under nonreducing conditions, Epo can be cross-linked in a molecular complex of 224 kDa on fetal liver cells, bone marrow cells, and on Friend virus-infected splenic erythroblasts. With the noncleavable cross-linker DSS, reduction of this complex results in two Epo conjugates of 136 kDa and 119 kDa as observed by others (2–6). These results provide evidence for a receptor consisting of two polypeptides covalently linked by a disulfide bond. These results differ from those of Sawyer et al. (2) and Todokoro et al. (4) who failed to observe Epo conjugates of greater than 136 kDa under nonreducing conditions using the Epo-dependent Friend virus-infected cell line SKT6, Friend virus erythroblasts, or plasma membranes derived from Friend virus-infected erythroblasts. Our observations support and extend other studies using mouse (3) and rat (6) fetal liver cells where 230–255-kDa Epo complexes were detected in the absence of reducing agents. The reason why the first two groups failed to observe disulfide-linked complexes under nonreducing con-
ditions is not clear, although the cell type used, the purity of the nonionic detergent used to solubilize cross-linked cells, and the use of isolated plasma membranes (2) may all have contributed to the difference, particularly if a labile disulfide linkage is involved. The present studies did not use nonionic detergents to solubilize cross-linked cell membrane proteins, but instead involved freeze-thawing and DNaS treatment of whole cells prior to SDS solubilization (14). This method may avoid potential problems associated with the use of nonionic detergents to separate solubilized membrane material including plasma membranes and lysosomes from nuclear-cytoskeletal residues that are then removed by centrifugation. In some experiments carried out under nonreducing conditions (Fig. 2A, lane 1), 136-kDa and 119-kDa bands were observed in addition to the 224-kDa band. The presence of these lower molecular weight bands appeared to be minimized by inclusion of iodoacetic acid to block free sulfhydryl groups (Fig. 2B, lane 1) suggesting that cell disruption may release substances capable of reducing labile disulfide linkages, a problem that may be exacerbated by the use of nonionic detergents. Studies with the insulin receptor (17) have suggested that a sulfhydryl component of the membrane which co-purifies with the insulin receptor can reduce specific disulfide bonds of the receptor during solubilization in SDS. In the present study, including oxidized glutathione prior to cell solubilization did not affect the amount of 224-kDa conjugate seen on nonreducing gels, and no conjugates greater than 224 kDa were observed in the resolving gel.2 In our experience, two problems may contribute to inconsistencies in demonstrating disulfide-linked proteins bound to Epo. The first relates to an observation that up to 50% of specifically bound 125I-Epo can remain associated with insoluble nuclear/cytoskeletal material following solubilization in 0.1–1% Triton X-100, and the second to the fact that with Triton-solubilized material analyzed under nonreducing conditions, most radioactivity did not enter the gel, whereas under reducing conditions, 136-kDa and 119-kDa bands were routinely observed. Similar observations have also been made by Sasaki et al. (3). In the present study, lower amounts of radioactivity were usually observed in the resolving gel of nonreduced compared with reduced samples (Figs. 2B and 5), although this difference appeared to be dependent on the time of Epo binding, at least with Friend virus erythroblasts (Fig. 5). The predominant Epo-binding proteins were calculated to have relative molecular masses of 190 kDa nonreduced, and 102 kDa and 85 kDa reduced (when the 34-kDa contribution of Epo was subtracted). Other smaller 125I-Epo binding proteins were also present which were more clearly resolved on 5–15% gradient SDS-polyacrylamide gels (Fig. 2). Thus, an 82-kDa/76-kDa doublet was observed under reducing conditions corresponding to a 90–74-kDa doublet nonreduced. The intermediate gradient lane showed that the 82-kDa species was derived from the 90-kDa nonreduced band, and the 76-kDa species from a 74-kDa nonreduced band (Fig. 2). Epo-receptor complexes very similar to those shown in Fig. 2 (lane 3) have also been demonstrated by Pekonen et al. (18) in human fetal liver cells. No direct relationship between the 82-kDa/76-kDa bands and the 136-kDa/119-kDa bands could be established by: 1) omitting protease inhibitors, 2) adding EGTA (1 mM) (Fig. 5), EDTA (up to 50 mM), or mercaptoethanol (up to 200 mM) prior to or during 125I-Epo binding or after DNase I treatment, 3) incubating cross-linked DNase-treated cells at 37°C for up to 2 h or by including trypsin (up to 0.67 mg/ml) in this incubation step, 4) varying the time of 125I-Epo binding to cells (Fig. 5), or 5) altering the 125I-Epo concentration 10-

2 P. J. A. McCaffery and M. V. Berridge, unpublished observations.

Appearance of the 224-kDa complex was maximum by 20 min and thereafter declined to low levels by 90 min. Because this change was not associated with the appearance of lower molecular mass bands under nonreducing conditions, and because these results could not be explained by rapid receptor internalization in the presence of sodium azide, it may be concluded that the 224-kDa complex was inefficiently solubilized at the 45- and 90-min time points. The results suggest that following Epo binding, the Epo receptor may become associated with cytoskeletal structures that are resistant to SDS solubilization.

A tentative structure for the Epo receptor based on present studies and those of others (2–6, 18) is proposed consisting of a complex of two polypeptides of 102 kDa and 85 kDa, covalently linked by a labile disulfide bond. Each polypeptide is closely associated with Epo to which it can be chemically cross-linked with equal efficiency. Other smaller polypeptides that can be cross-linked to Epo exhibit kinetics of Epo binding that are distinct from those of the heterodimeric complex and are not considered to be part of the functional binding complex. This structure, if confirmed, would be unique among receptors for hematopoietic growth factors, none of which are known to contain disulfide bonds. Rather, the Epo receptor warrants comparison with a primitive family of membrane receptors including those for insulin, epidermal growth factor, nerve growth factor, and insulin-like growth factor which bear sequence and structural homologies (17, 19–21), and whose component polypeptides, some of which are disulfide-bonded, associated cooperatively in the cell membrane, either covalently or noncovalently to form high affinity receptors for their respective ligands (21, 22). It is of interest to speculate that the Epo receptor heterodimer may also associate in the membrane to form a receptor complex analogous to the insulin receptor. In this context, two affinity classes of Epo receptors have been described by several groups (4, 23, 24), although in the present study (Fig. 6) and in studies by several other groups (3, 4, 6, 25), only a single affinity class of Epo binding site has been reported.

REFERENCES

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