Subunit Structure of the Erythropoietin Receptor Analyzed By $^{125}$I-Epo Cross-Linking in Cells Expressing Wild-Type or Mutant Receptors

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To analyze the structure of the murine erythropoietin receptor (EpoR), wild-type or mutant EpoR cDNAs were expressed in cell lines, and the proteins that cross-linked with $^{125}$I-labeled erythropoietin (Epo) were analyzed by immunoprecipitation using an antibody against the intracellular region of the cloned EpoR. COS-7 cell transfectants expressing the wild-type EpoR showed two major cross-linked species of 145 and 110 Kd, both of which were recognized by the antibody against the cloned EpoR after denaturation under reducing conditions. Furthermore, a reduction in sizes of both cross-linked bands was observed in COS-7 transfectants expressing a mutant receptor with an internal deletion, thus indicating that both species contain the cloned EpoR. COS-7 cells expressing mutant receptors with carboxy-terminal deletions showed cross-linked bands corresponding to the smaller species of the two observed in cells expressing the wild-type receptor. In contrast to COS-7 cell transfectants, DA3 cells expressing wild-type or mutant EpoR cDNAs showed an additional cross-linked species of 130 Kd. The size of this species was not altered by deletions in EpoR, showing that it did not contain EpoR. The 130-Kd cross-linked band, which would contain a 95-Kd protein, was also observed in a murine erythroleukemia cell line, D1B. These results suggest that Epo associates with a second component of 95 Kd, which is specifically expressed in hematopoietic cells.

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MATERIALS AND METHODS

Cells and reagents. An IL-3-dependent cell line DA3$^{31}$ and subclones of this cell line expressing the wild-type and mutant EpoRs were maintained as previously described.$^{11}$ A murine erythroleukemia cell line, D1B, was obtained from American Type Tissue Collection (ATCC) and grown according to the protocol provided. COS-7 cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (FBS). An expression plasmid for the murine EpoR, pXM-Epo$^{2}$, was kindly provided by A. D’Andrea (Harvard University, Cambridge, MA). Expression plasmids for the EpoR with various deletions were described previously.$^{11}$ A rabbit antiserum against the cytoplasmic region of the murine EpoR was raised against a bacterial fusion protein that contains the intracellular portion of the EpoR.

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Fig 1. Cross-linking of 125I-Epo to COS-7 cells expressing the wild-type or mutant EpoRs. COS-7 transfectants expressing the wild-type (lanes 1 through 3), H-mutant (lane 4), S-mutant (lane 5), or PB-mutant (lane 6) EpoR were incubated with 125I-Epo in the presence or absence of an excess of unlabeled Epo as indicated below the panel. Cross-linking reaction was performed by adding a cross-linker, disuccinimidyl suberate, except for a negative control (lane 2). The EpoR was immunoprecipitated by anti-EpoR antibody and subjected to SDS-PAGE and autoradiography. Positions of size standards are shown at the right margin of the gel.

region of the EpoR. Recombinant human Epo was kindly provided by Amgen Biological (Thousand Oaks, CA). All other reagents used were purchased from commercial sources.

Cross-linking and immunoprecipitation. Iodination of Epo and bioassay of labeled Epo were performed as previously described. In brief, carrier-free recombinant human Epo was iodinated with 125I using IODO-BEADS (Pierce, Rockford, IL). After purification by chromatography, the bioactivity of the labeled Epo was measured by [3H]thymidine-incorporation assay using an Epo-responsive cell line, DA3/EpoR-Wt. This procedure provided Epo with 0.8 atom of 125I per molecule (48 mCi/μg of protein).

For cross-linking experiments in COS-7 cells, 10 μg of an expression plasmid for the wild-type murine EpoR or one of various mutant EpoRs were transfected into COS-7 cells by the diethyl aminoethyl (DEAE)-dextran-mediated method for transient expression as described. For eighty hours after transfection, cells were incubated with 2 nmol/L of 125I-Epo for 1 hour at 37°C in the binding medium previously described for the binding assay. After washing three times in cold phosphate-buffered saline (PBS), cells were incubated with 500 μmol/L of disuccinimidyl suberate in cold PBS for 20 minutes on ice. Cross-linking reaction was terminated by adding 1 mol/L tris-HCl to the final concentration of 50 mmol/L. Cells were then washed three times in cold tris-buffered saline (20 mmol/L tris-HCl, 150 mmol/L NaCl) and lysed in the lysis buffer (1% Triton X-100, 20 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and aprotinin) as described previously. The EpoR was then immunoprecipitated from cell lysates as described previously except that the rabbit antiserum against the cytoplasmic domain of the EpoR was used instead of that against the amino-terminus of the receptor. Immunoprecipitates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography.

For double immunoprecipitation of the EpoR cross-linked with 125I-Epo, immunoprecipitates prepared as described above were first heated at 100°C for 5 minutes in 0.05 mL of 1× SDS sample buffer containing 5% 2-mercaptoethanol. The EpoR eluted from protein A-agarose beads was diluted to the final volume of 2 mL with the lysis buffer containing 0.1% bovine serum albumin and then concentrated to about 0.05 mL using Centricon 30 (Amicon, Danvers, MA). SDS and 2-mercaptoethanol were washed away from the eluates two more times by dilution with the lysis buffer and concentration by Centricon 30. After a final dilution to 1 mL with the lysis buffer, the EpoR was immunoprecipitated again using the same anti-EpoR serum.

Cross-linking experiments in DA3 cells or D1B cells were performed essentially as described above for COS-7 cells.

125I-Epo binding assay. The expression plasmids for the wild-type EpoR or H-mutant EpoR were transfected into COS-7 cells grown in 10-cm plates as described above. At 24 hours after transfection, transfected cells were replated on 24-well plates. At 48 hours after transfection, cells in each well were washed three times with the binding medium and then incubated at 37°C for 90 minutes in 0.1
cells were washed three times with cold PBS and lysed in the lysis buffer described above. The radioactivity of the cell lysates and the binding media clarified by centrifugation was measured by a γ counter. Specific binding was calculated by subtracting the radioactivity associated with the cells in the presence of unlabeled Epo (nonspecific binding) from that in the absence of unlabeled Epo (total binding). The number and the affinity of Epo binding sites were determined by the Scatchard plot analysis from duplicate samples.

RESULTS

125I-Epo cross-linking studies in COS-7 cells expressing the wild-type or various mutant EpoRs. To analyze the relationship between 125I-Epo cross-linked species, the cloned EpoR wild-type and mutant EpoRs were expressed in COS-7 cells and subjected to cross-linking studies using 125I-Epo. As shown in Fig 1, lane 3, two cross-linked species were evident with molecular masses of 110 Kd and 145 Kd in cells expressing the wild-type EpoR. This result is consistent with that of D’Andrea et al12 who showed two cross-linked bands of 140 Kd and 100 Kd in COS-7 cell transfectants expressing the cloned EpoR. COS-7 cells expressing the PB mutant of EpoR, which has an internal deletion of 20 amino acids in the intracellular region,11 showed a slight reduction in sizes of the two bands as shown in Fig 1, lane 6. This is also demonstrated in other experiments (see Fig 4A, compare lanes 1 and 2). These results suggest that both of the cross-linked species contain the EpoR expressed from the transfected cDNA. In contrast, the H- or S-mutant EpoRs, which have carboxy-terminal truncations of 108 or 146 amino acids, respectively,12 gave rise to single cross-linked bands (Fig 1).

Based on the sizes of the mutants, the bands correspond to the smaller band of the two observed with the wild-type receptor. In addition to these bands, a 70-Kd band was observed in all cells expressing EpoRs. This band is most probably a cross-linked dimer of 125I-Epo, since its apparent molecular mass is unaffected by deletions of the receptor and corresponds to that expected for Epo dimers.

To further confirm that the cloned EpoR is contained in the two cross-linked species, we analyzed them by double immunoprecipitation using an antiserum against the intracellular portion of the EpoR as described in Materials and Methods. As shown in Fig 2, lane 4, both the 140- and 100-Kd species were immunoprecipitated with the EpoR antibody after denaturation in the presence of SDS and 2-mercaptoethanol. This result indicates that both contain EpoR and were not co-precipitated because of covalent or noncovalent interactions with the other.

COS-7 cell transfectants expressing the wild-type EpoR have been shown to exhibit two affinity classes of Epo binding sites,7 although transfected hematopoietic cell lines routinely show only a single class of high-affinity receptors.2 Thus, we examined the possible correlation between the number of affinity classes and the cross-linked bands. For this purpose, the affinities of the wild-type and H-mutant EpoRs expressed in COS-7 cells were measured by the Scatchard plot analysis. The results are shown in Fig 3 and demonstrate that both receptors exhibited a single class of binding sites with comparable affinities. The basis for the absence of a second affinity site is not known. Irrespective, the presence of the additional Epo–cross-linked species observed for the wild-type receptor had no effect on the affinity of the receptor.

125I-Epo cross-linking studies in hematopoietic cell lines expressing the EpoR. COS-7 cells may lack a component that is required for forming the functional EpoR, because expression of the cloned EpoR in COS-7 cells does not change the growth kinetics of the cells when cultured in EPO23 nor does EPO induce tyrosine phosphorylation in these cells comparable with that seen in myeloid lineage cells (O. Miura and J.N. Ihle, unpublished data, April 1992). Thus, we examined, by cross-linking with 125I-Epo, subclones of the IL-3–dependent cell line, DA3, expressing the wild-type or mutant EpoRs. DA3 transfectants expressing the wild-type EpoR are as responsive to Epo as they are to IL-3.11 DA3 cells expressing the wild-type receptor showed a 130-Kd cross-linked band in addition to the other bands observed in COS-7 cell transfectants (Fig 4A, lane 1). No cross-linked bands were detected when parental cells were used (data not shown).
The extra band, with an apparent molecular mass of 130 Kd, was also observed in DA3 cells expressing the H- or PB-mutant of the EpoR (Fig 4A, lanes 2 and 3). Because this species was not affected by deletions in the EpoR cDNA and was absent in COS-7 cell transfectants expressing the cloned EpoR, it is speculated that this species represents cross-linking of Epo to a component other than EpoR. We also examined a murine erythroleukemia cell line, D1B, which expresses the endogenous EpoR. As shown in Fig 4B, lane 3, this cell line exhibited the three cross-linked species whose sizes are the same as those observed in DA3 cells expressing the wild-type EpoR, although the 110-Kd band was very faint. Thus,

Fig 3. Scatchard plot analysis of COS-7 cells expressing the wild-type or H-mutant EpoR. COS-7 transfectants expressing the wild-type (A and C) or H-mutant (B and D) EpoR were examined for the Epo-specific binding sites as described in Materials and Methods. Binding curves for total (□ — □), nonspecific (○ — ○), and specific (△ — △) bindings are shown in panels A and B. The binding data were then plotted and analyzed by the method of Scatchard (C and D).

Fig 4. Cross-linking of 125I-Epo to hematopoietic lines expressing the EpoR. (A) DA3 cells expressing the wild-type (lane 1), PB-mutant (lane 2), or H-mutant (lane 3) EpoR were analyzed by cross-linking with 125I-Epo. An arrow indicates the 130-Kd band that was not observed in COS-7 transfectants. (B) DA3 cells expressing the wild-type (lane 1) or H-mutant (lane 2) EpoR and D1B cells (lane 3), a murine erythroleukemia cell line, were analyzed by cross-linking with 125I-Epo in the presence (lane 1) or absence (lanes 2 and 3) of a saturating concentration of unlabeled Epo. Three arrows indicate 145-Kd, 130-Kd, and 110-Kd bands observed in D1B cells.
the pattern of the cross-linked bands in D1B is consistent with those previously observed with various erythroid progenitor cells and cell lines.

**DISCUSSION**

These studies were directed at further characterizing the protein complexes that are seen in cross-linking studies of Epo with the cloned EpoR. Our results show the existence of three distinct cross-linked species of 145, 130 and 110 Kd consistent with previous studies. The 145- and 110-Kd complexes are seen in hematopoietic cells and in COS-7 cells expressing the cloned EpoR. The 130-Kd complex is uniquely seen in hematopoietic cells.

The 145- and 110-Kd complexes contain the protein encoded for by the cloned EpoR as shown by changes in the sizes of the complexes when various deleted or truncated mutants are expressed in either DA3 cells or in COS-7 cells. In addition, both complexes were immunoprecipitated with antisera against EpoR after reduction and denaturation, indicating that they did not contain a tightly associating protein. The cloned EpoR has a predicted size of 55 Kd and has been shown to be processed to forms of 62, 64, and 66 Kd. The predominant form that is found on the cell surface and that rapidly becomes tyrosine phosphorylated following Epo binding, is a further processed form of 72 Kd. Thus, the predicted size of a complex containing the cell surface EpoR binding, is a further processed form of 72 Kd. The cloned EpoR has been shown to be processed to forms of 62, 64, and 66 Kd. The predominant form that is found on the cell surface and that rapidly becomes tyrosine phosphorylated following Epo binding, is a further processed form of 72 Kd. Thus, the predicted size of a complex containing the cell surface EpoR binding, is a further processed form of 72 Kd.

The additional 35-Kd component could be Epo and be caused by the binding of Epo dimers to EpoR. Alternatively, the 35-Kd component may be a unique component. The latter possibility is indicated by the observation that with the two carboxyl-truncated mutants, a complex with a reduced size consistent with the 110-Kd complex is seen; however, a complex with the expected reduced size of the 145-Kd complex is not seen. Thus, in the absence of the carboxyl region of the receptor, the additional 35-Kd component is not associated with EpoR. Because these mutants have been shown to have comparable Epo binding with that of the wild-type receptor, it is unlikely that the differences are related to binding of Epo or Epo dimers. The requirement for the carboxyl domain suggests that this region is either the site of interaction with a 35-Kd component or the deletion of this region affects the structure of EpoR in a way that eliminates the association of the 35-Kd component. Because comparable results are seen in both DA-3 cells and COS-7 cells, the 35-Kd component is not specifically expressed in hematopoietic cells. Lastly, because the H-mutant is mitogenically active and a complex with a size consistent with the presence of the additional 35-Kd component is not seen, the association of the 55-Kd component with EpoR is not required for mitogenic activity.

Recent studies suggested that Epo cross-linked complexes that would correspond to our 130- and 145-Kd complexes did not contain EpoR. This was based on the inability of an antiserum against EpoR to immunoprecipitate the two complexes following reduction and denaturation. The lack of reactivity of the 130-Kd complex is consistent with our data; however, the lack of reactivity of 145-Kd complex is in distinct contrast to our data. It is also inconsistent with the changes in sizes of this complex that are seen with different truncated mutants. It is possible that in the studies of Mayeux et al., the antiserum against EpoR that was used did not efficiently precipitate the reduced and denatured receptor.

Our studies also show the unique existence of an Epo-containing complex of 130 Kd in the hematopoietic cells examined. The predicted size of the protein that is cross-linked with Epo is 95 Kd. The 130-Kd complex does not contain EpoR because the size of the complex does not change when truncated EpoRs are expressed. However, the complex associates with EpoR because it is specifically immunoprecipitated with antisera against EpoR. The 130-Kd Epo complex is not seen in cross-linking studies with the parental DA3 cells (data not shown). Thus, the data are most consistent with the existence of a 95-Kd protein that associates with the EpoR on the cell surface and acquires the ability to bind to Epo. The formation of heterodimers in the presence of ligand has been shown for many receptors of the cytokine receptor family.

Previous studies have suggested the presence of proteins that associate with the EpoR of 130 Kd or 97 to 100 Kd. The 130-Kd protein was detected as a phosphate-labeled protein obtained by incubating cells with biotinylated Epo, cross-linking with a thiol-cleavable cross-linker, isolation of complexes by avidin-agarose, and labeling with ATP. This component was not detected in our cross-linking studies.

The presence of a 97-Kd phosphoprotein that co-immunoprecipitated with EpoR has been reported and previous studies have shown that one of the substrates of tyrosine phosphorylation after Epo stimulation is a 92 to 100 Kd protein. Whether the 97-Kd protein is related to the 95-Kd protein associated with Epo in our studies is not known but appears to be unlikely. In particular, we have found that the only phosphotyrosine-containing protein detected in immunoprecipitates of EpoR is the receptor and specifically, a 97-Kd protein was not detected. Thus, the 95-Kd protein in the 130-Kd complex does not become phosphorylated and therefore it is unlikely to be the 97-, 92-, to 100-Kd phosphoproteins detected in other studies. We have previously shown that induction of tyrosine phosphorylation plays a critical role in the signal transduction from the cloned EpoR, which lacks a tyrosine-kinase domain. Thus, it is tempting to speculate that the 95-Kd protein may be the tyrosine kinase that interacts with the cloned EpoR. Further characterization and cDNA cloning of the 95-Kd component will be required to address this possibility and to provide further insights into the signal transduction mechanisms of the EpoR.

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**REFERENCES**

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