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LIGAND BINDING KINETICS OF A SOLUBLE FULL-LENGTH MURINE ERYTHROPOIETIN RECEPTOR

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Summary. The full-length murine erythropoietin receptor was expressed in Sf9 cells using a baculovirus vector. Erythropoietin receptors in solubilized Sf9 cell lysates bound erythropoietin with high affinity (92 pM). Erythropoietin receptor-^{125}I-labeled erythropoietin association and dissociation kinetics using solubilized Sf9 cell lysates revealed a ka of 0.16 nM^{-1} min^{-1} and a kd of 0.00055 min^{-1} giving an observed KD of 3.45 pM. The erythropoietin receptor was partially purified from Sf9 cell lysates by chromatography on Con A Sepharose. When erythropoietin receptors were crosslinked to ^{125}I-labeled erythropoietin and analyzed by SDS-7.5% PAGE protein complexes of 90 and 125 kDa were observed with receptors in solubilized lysate, and 170 and 190 kDa with the partially purified receptors.

The erythropoietin receptor is a member of the hematopoietic growth factor receptor family (1). The receptors comprising this family (IL-2ß, IL-3, IL-4, IL-5, IL-6, IL-7, LIF, GM-CSF, G-CSF, oncostatin M, CNTF, growth hormone and prolactin) have a single membrane-spanning domain, four positionally conserved cysteines and a Trp-Ser-X-Trp-Ser motif in their extracytoplasmic domain, and lack a kinase motif in their cytoplasmic domain. Another characteristic of this receptor family is the formation of multimeric complexes following ligand binding. These receptor complexes, which influence binding affinity (2), may be heteroligomers ((IL-2 (3), IL-3 (4), IL-4 (5), IL-5 (6), IL-6 (7), IL-7 (8), GM-CSF (9), LIF, oncostatin M, CNTF (10)) or homodimers ((G-CSF (11) and growth hormone (12)). Although both the gene for the erythropoietin receptor (13) and its ligand, erythropoietin (14,15), have been cloned, the nature of the receptor-ligand interaction for this protein pair is not completely understood. Hydrodynamic studies of solubilized, native, erythropoietin-erythropoietin receptor complexes (16) as well as studies employing bifunctional crosslinkers (17) suggest that receptor-ligand binding is associated with multimer formation, but the actual components of such multimers have not been identified. Mutation of arginine 129 to cysteine in the amino terminal domain of the erythropoietin receptor results in spontaneous homodimerization and ligand-independent receptor signaling (18). Spontaneous ligand-independent homodimerization has also been observed with transfected and mutated erythropoietin receptors (19).

Attempts to define the nature of the erythropoietin-erythropoietin receptor interaction have been hindered by the low number of receptors expressed by erythroid progenitor cells (20). Expression of the extracyto-

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plasmic domain of the human erythropoietin receptor both as a soluble protein (21,22) and a fusion protein (23) has been achieved, but the yields have been low for the monomeric complexes (21,22) in contrast to what is observed with the soluble extracytoplasmic domain of the growth hormone receptor (12). To facilitate the analysis of erythropoietin receptor-ligand interactions, we have employed an insect cell-baculovirus expression system (24) which has the advantages of providing large quantities of protein and also of being eukaryotic. With this system, we have been able to partially purify the full length murine erythropoietin receptor with a single affinity chromatography procedure and to study the kinetics of the receptor-ligand interaction in solution.

Methods

Production of the Full-Length Murine Erythropoietin Receptor in Sf9 Cells

Sf9 cells in suspension culture (2 x 10^6 cells/ml) were infected at an m.o.i. of 10 with a recombinant baculovirus containing the full length murine erythropoietin receptor cDNA (25). Preliminary studies employing ^{35}S-methionine-cysteine metabolic labeling and Western analysis revealed that receptor protein production was maximal 48 hours after infection, and the cells were harvested at that time by centrifugation at 400 g for 5 minutes. To obtain solubilized erythropoietin receptors, the cell pellet was resuspended at a concentration of 10^7 cells/ml in ice cold lysis buffer (10 mM sodium phosphate, pH 7.4, containing 10% glycerol, 0.6% CHAPS, 0.5 mM DTT 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 8 μg/ml pepstatin and 2 μg/ml leupeptin) and rocked for one hour at 4°C. The suspension was clarified by centrifugation at 10,000 g for 15 minutes and the pellet discarded.

Affinity Chromatography of the Solubilized Erythropoietin Receptor

For affinity chromatography using Con A-Sepharose (Pharmacia), the beads were equilibrated with 10 mM Tris-Cl pH 7.5, 150 mM NaCl (TBS) containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$. Two ml of lysate adjusted to a protein content of 1.5 mg/ml with lysis buffer were incubated with 100 μl of packed Con A Sepharose beads at 4°C for 12 hr. The beads were washed five times with 1 ml of TBS and the adherent proteins eluted with 0.5 M α methyl mannoside in TBS containing 1 mM DTT, 0.5 mM EDTA, 0.06% CHAPS and 1 mM PMSF.

Equilibrium Binding Studies

For whole cell lysate-ligand equilibrium binding studies, 100 μg of lysate protein was incubated with ^{125}I-labeled erythropoietin in a final volume of 100 μl in 50 mM Tris-Cl, pH 7.5, at 4°C for 3 hours in 500 μl microfuge tubes with addition of a 1000-fold concentration of unlabeled erythropoietin to some tubes for assessment of nonspecific binding. The ligand-receptor complexes were separated from unbound protein by precipitation with PEG (final concentration 10%) and 250 μg of bovine gamma globulin at 4°C for 20 minutes (26). The precipitates were collected by centrifugation through dibutyl phthalate oil at 10,000 g for 10 minutes at 4°C for gamma scintillation counting. Binding isotherms were analyzed using the LIGAND program (27).

Association and Dissociation Kinetics

Receptor-ligand association and dissociation kinetics were examined using whole cell lysates. For association kinetics, 100 μg of lysate protein was incubated with 8 ng of ^{125}I-erythropoietin at 4°C with or without a 1000-fold excess of unlabeled erythropoietin. At selected time intervals, bound ligand was separated from unbound ligand by PEG precipitation as described above. The association rate constant was calculated using a pseudo-first order reaction equation to linearize the data, and the ka was derived from the k observed according to the equation $ka = \frac{k\ observed}{[L]}$ where L is the free ligand concentration, and the k observed is derived from the slope of the pseudo-first order rate plot of ln(Beq/Beq-B) where Beq is the amount of bound ligand at the steady-state level and B is the amount bound at a given time (28). For receptor-ligand dissociation studies, 100 μg of lysate protein was incubated with 8 ng of ^{125}I-erythropoietin at 4°C in the presence or absence of a 1000-fold excess of unlabeled erythropoietin. After three hours of incubation, excess unlabeled erythropoietin was added to the set of microfuge tubes containing lysate and ^{125}I-erythropoietin and, at selected time intervals,

bound ligand was separated from free ligand by PEG precipitation. A series of parallel tubes containing whole cell lysate and ^{125}I-erythropoietin was carried through the whole incubation period to monitor ligand degradation. The dissociation rate constant, kd, was calculated from the slope of a plot of ln(B/Beq) versus time where B is the amount of ligand bound at a given time and Beq the amount bound at the steady-state level (29).

Crosslinking of Erythropoietin Receptors

Solubilized receptors in whole cell lysates or following purification by affinity chromatography as described above were crosslinked to ^{125}I-erythropoietin by exposure to 400 μM DSS at 4°C for one hour after prior exposure to ^{125}I-erythropoietin (7.5 ng) alone or in the presence of 1000-fold excess of unlabeled erythropoietin for three hours. The reaction was quenched by the addition of Tris-Cl pH 8.0 to a final concentration of 150 mM, and the crosslinked proteins were directly dissolved in SDS sample buffer. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 7.5% gels according to the technique of Laemmli (30).

Iodination of Recombinant Erythropoietin

Recombinant erythropoietin was iodinated to a specific activity of 31-154 μCi/μg using the Iodogen technique (31). Biologic activity was preserved as established by an in vitro bioassay using an erythropoietin-dependent erythroleukemia cell line (32), and all preparations were analyzed by SDS-PAGE to document the purity and integrity of the labeled protein.

Protein Assay

Protein content was assayed using the bicinchoninic acid technique (Pierce) with BSA as the protein standard (33).

Results and Discussion

Detergent solubilization of Sf9 cells infected with a recombinant baculovirus containing the cDNA for the full length murine erythropoietin receptor yielded biologically active receptor protein. Erythropoietin receptors in solubilized Sf9 cell lysates bound ^{125}I-erythropoietin with high affinity (92 pM) (N= 10). A typical equilibrium binding isotherm is shown in Figure 1.

For comparison, binding of ^{125}I-erythropoietin by intact erythroid progenitor cells revealed both high (90 pM) and low (570 pM) affinity sites (34), while only low affinity binding (1.5 nM) of the hormone was observed with immobilized exoplasmic domain of the receptor (23), and the affinity of soluble erythropoietin receptors containing only the exoplasmic domain ranged from 1.1 to 13 nM (21,22). These data indicate that not only does the plasma membrane impose constraints with respect to receptor-ligand interactions but that immobilization on a solid matrix or loss of the cytoplasmic domain does also. The data also indicate that high affinity ligand binding by the full length erythropoietin receptor does not require accessory proteins.

Since high affinity binding of erythropoietin was possible employing the full length erythropoietin receptor in solubilized, infected Sf9 cell lysates, we analyzed receptor-ligand association and dissociation kinetics. Representative association and dissociation curves are shown in Figure 2A and B, and the corresponding rate constants for the initial linear portion of these curves are shown in Figure 2C and D. The measured ka was 0.16 nM^{-1} min^{-1} and the kd was 0.00055 min^{-1} giving an observed KD of 3.45 pM (N = 3) which is higher than the value calculated from equilibrium binding studies. The data indicate a rapid on rate and a slow off rate which is in keeping with the behavior of a receptor normally exposed to low concentrations of its cognate ligand. The results, however, differ from those obtained by Harris et al (ka

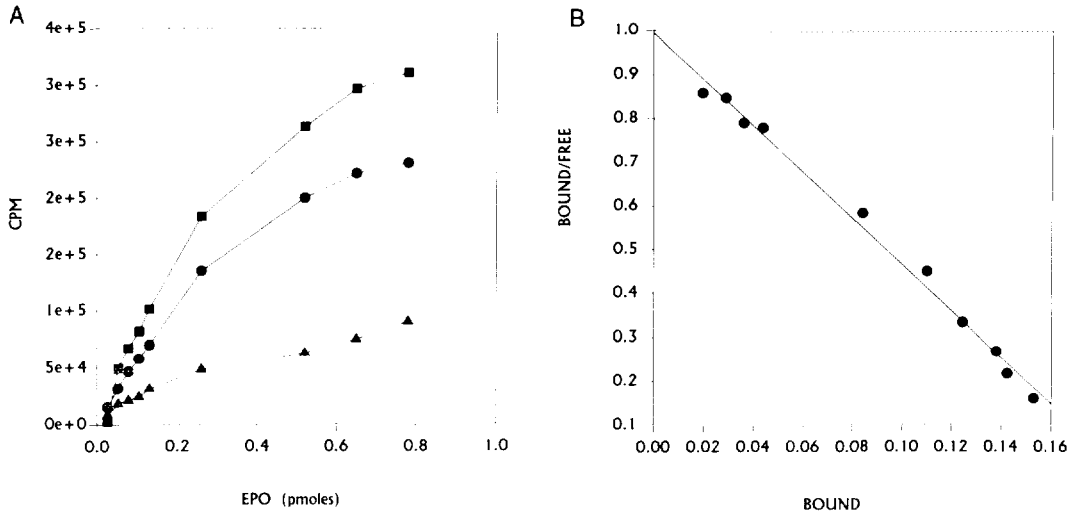

Figure 1. Equilibrium binding of ^{125}I-erythropoietin by solubilized murine erythropoietin receptors expressed in Sf9 cells A) One hundred µg of solubilized whole cell lysates from Sf9 cells infected 48 hours before with a recombinant baculovirus containing the cDNA for the full-length murine erythropoietin receptor was incubated with varying concentrations of ^{125}I-erythropoietin for 3 hours at 4°C in the presence or absence of unlabeled erythropoietin as described in Methods. The ligand-receptor complexes were separated by PEG precipitation for gamma scintillation counting. ■ total binding; ● specific binding; ▲ nonspecific binding . B) Scatchard analysis of the equilibrium binding data obtained in A.

= 3.5 nM^{-1} min^{-1}, kd = 1.06 min^{-1} and KD = 310 pM) using the immobilized extracytoplasmic domain of the receptor (23) and the kd of 0.02-0.04 nm^{-1} obtained by Hilton et al for murine erythropoietin receptors expressed at the cell surface by transfected COS cells (35), and demonstrate that the ligand-binding behavior of a receptor is influenced by its conformational status.

To further examine the receptor-ligand interaction, we purified the erythropoietin receptor from infected Sf9 cell lysate. Since N glycosylation of mammalian proteins by Sf9 cells primarily involves the addition of mannose residues (36), we employed affinity chromatography using immobilized Con A. As shown in Figure 3, a single affinity step was sufficient to highly purify the baculovirus-expressed receptor. However, as has been previously observed (37), the ligand-binding kinetics of the purified receptors could not be studied since these receptor-ligand complexes did not precipitate in the presence of PEG. It was, however, possible to perform crosslinking studies with these complexes. As shown in Figure 4, when ^{125}I-erythropoietin was incubated with infected Sf9 cell lysates and crosslinked with DSS, protein complexes of 100 and 125 kDa were observed (lanes 1 and 5) which were due to the crosslinking of ^{125}I-erythropoietin (apparent MW with SDS-7.5%PAGE 35 kDa) to proteins of 90 and 65 kDa. Similar observations have been made following expression of the full length erythropoietin receptor in COS cells (13). However, when Con A Sepharose-purified receptors were incubated with ^{125}I-erythropoietin and exposed to the crosslinking agent, complexes with molecular weights of approximately 170 and 190 kDa were observed (lanes 6 and 7). These represented proteins which specifically bound labeled erythropoietin since

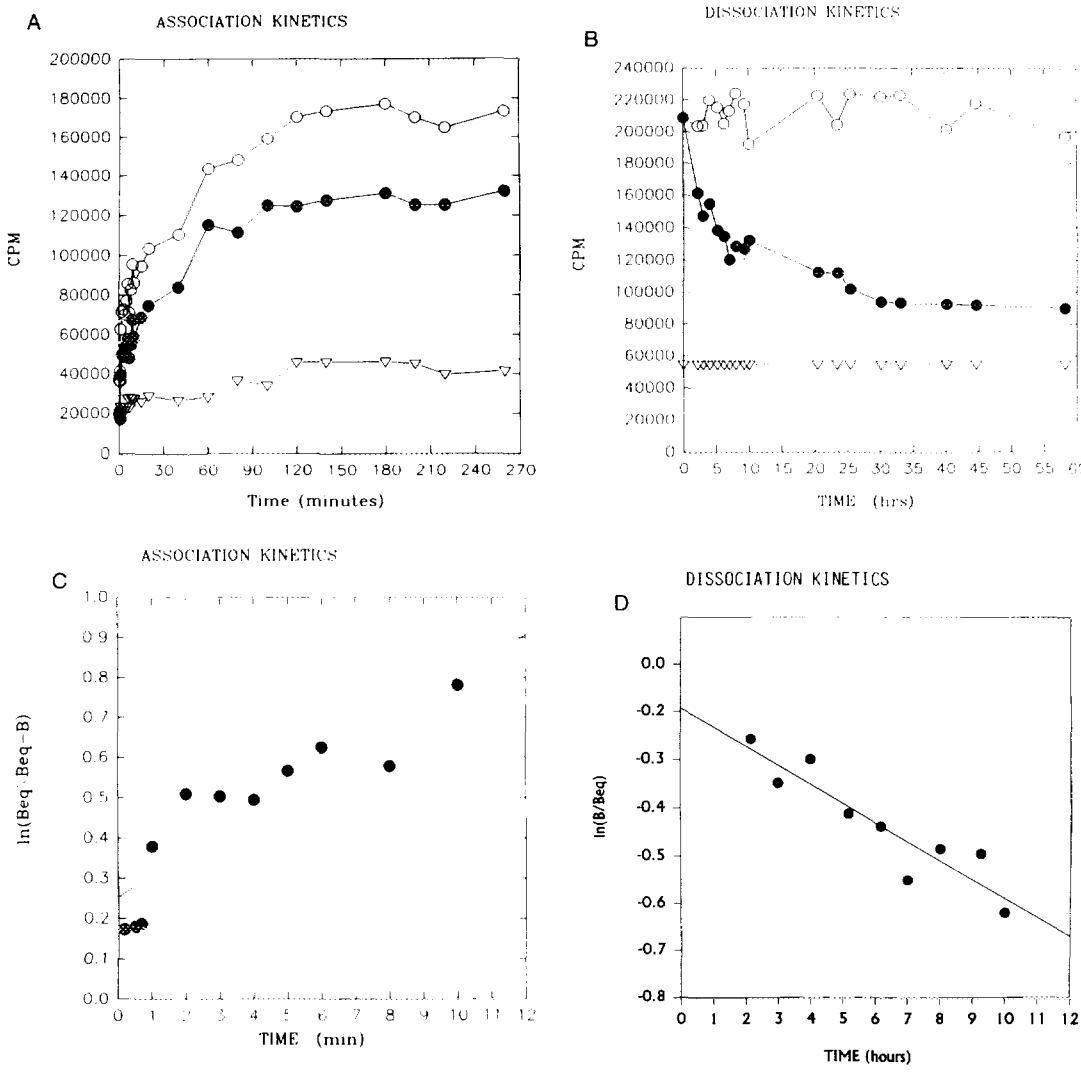

Figure 2. Association and dissociation kinetics for ^{125}I-erythropoietin binding to whole cell lysates of infected Sf9 cells. A) For association kinetics studies, aliquots of whole cell lysates were incubated with ^{125}I-erythropoietin at 4°C in the presence or absence of excess unlabeled erythropoietin for the indicated time intervals as described in Methods. B) For dissociation kinetic studies, aliquots of whole cell lysates were incubated with ^{125}I-erythropoietin at 4°C for three hours in the presence or absence of excess unlabeled erythropoietin and, thereafter, excess unlabeled erythropoietin was added to a set of samples containing only ^{125}I-erythropoietin as described in Methods. O total ^{125}I-erythropoietin binding; • specific binding in the presence of unlabelled ligand for the indicated time period; and nonspecific binding when unlabelled ligand was present from the start of the incubation period. C) Pseudo-first order association rate plot for the data in A) where Beq is the amount of ligand bound at equilibrium and B is the amount bound at the indicated time. D) Dissociation rate plot for the data in B) where Beq is the amount of ligand bound at equilibrium and B, the amount bound at the indicated time.

binding was competed for by unlabeled erythropoietin. Whether these complexes represent two receptors complexed with one or two erythropoietin molecules or a heteroligomeric complex is unknown. However, this is the first direct

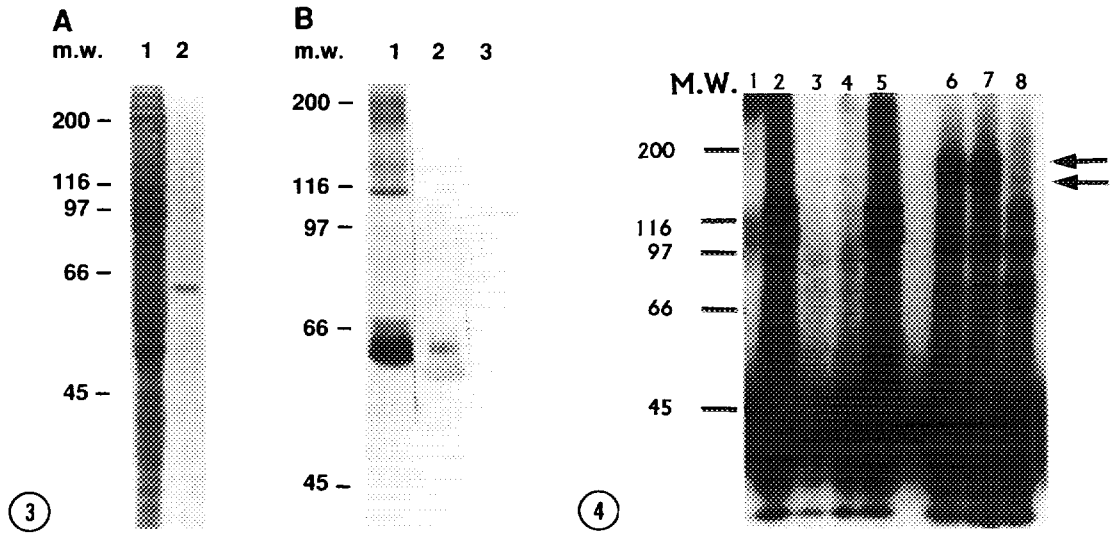

Figure 3. Purification of the full-length murine erythropoietin-receptor from Sf9 cells infected with a recombinant baculovirus containing the receptor cDNA. Solubilized whole cell lysates were chromatographed on Con A Sepharose as described in Methods, and the starting material and column eluate were subjected to SDS-PAGE and Western blotting using an antiserum to the C-terminal domain of the receptor. A) Lanes 1) Sf9 whole cell lysate (100 μg) and 2) Con A Sepharose eluate (100 μg) after SDS-7.5% PAGE and Coomassie blue staining. B Lane 1) Sf9 whole cell lysate and 2) and 3) Con A Sepharose eluate following SDS-7.5% PAGE and Western blotting in the absence 2) or presence 3) of soluble receptor protein.

Figure 4. Crosslinking of ^{125}I-erythropoietin to soluble erythropoietin receptors isolated by affinity chromatography using Con A Sepharose. Varying concentrations of receptor protein were incubated at 4°C for 3 hours with 7.5 ng of ^{125}I-erythropoietin in the absence or presence of excess unlabeled erythropoietin before crosslinking with DSS. For comparison, similar studies were performed simultaneously with whole cell lysates from infected and noninfected cells. Lane 1) ^{125}I-erythropoietin alone; Lanes 2 and 5) ^{125}I-erythropoietin and infected whole cell lysate; Lane 3) ^{125}I-erythropoietin, unlabeled erythropoietin and infected whole cell lysate; Lane 4 ^{125}I-erythropoietin and noninfected whole cell lysate; Lanes 6 and 7) ^{125}I-erythropoietin and 4.7 or 9.4 μg of Con-A purified lysate; Lane 8) ^{125}I-erythropoietin, unlabeled erythropoietin and 4.7 μg Con-A purified lysate. The arrows indicate the position of the receptor dimers.

demonstration of erythropoietin-induced receptor dimerization or oligomerization. Failure to observe receptor oligomerization in Sf9 whole cell lysates probably represents a "prozone" phenomenon.

In summary, the Sf9 insect cell-baculovirus expression system provides the opportunity to obtain large quantities of biologically active receptor in an easily purified form. The ability to obtain large quantities of pure, biologically active receptor protein should facilitate future studies directed at defining receptor-ligand interactions and the mechanisms involved in oligomerization of the erythropoietin receptor.

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