Isolation of the Full-Length Murine Erythropoietin Receptor Using a Baculovirus Expression System

By Jerry L. Spivak, Lena S. Avedissian, Jacalyn H. Pierce, Donna Williams, W. David Hankins, and Roy A. Jensen

The full-length murine erythropoietin receptor was expressed in Spodoptera frugiperda (Sf9) cells using a recombinant baculovirus vector. Erythropoietin receptor protein production was maximal 48 hours after infection, as determined by metabolic labeling and immunoblotting; receptor protein varied in molecular mass from 62 to 76 kD. Erythropoietin receptors produced in Sf9 cells could be solubilized using CHAPS in a form capable of binding erythropoietin, and the solubilized receptor bound to immobilized Concanavalin A (Con A) and wheat germ agglutinin, as well as to immobilized recombinant human erythropoietin. Analysis of the distribution of erythropoietin receptors in Sf9 plasma membrane and cytosol fractions using lectin affinity chromatography revealed that membrane-bound receptor had a higher apparent molecular mass and contained the bulk of receptors that bound to wheat germ agglutinin. The receptor was purified by sequential affinity chromatography.

ERYTHROPOIESIS is regulated by the glycoprotein hormone, erythropoietin (reviewed in Krantz'). Erythropoietin promotes proliferation of erythroid progenitor cells and maintains their viability by interacting with specific surface receptors expressed by these cells.2,3 However, although the genes for both erythropoietin5,6 and its receptor7 have been cloned, the nature of the receptor-ligand interaction is not fully understood, while the mechanisms involved in erythropoietin receptor-mediated signal transduction are being rapidly defined.7,12

The erythropoietin receptor is a member of the hematopoietic growth factor receptor superfamily, which is characterized by the presence of four conserved cysteine residues and a WSXWS box in the N-terminal domain.13 The formation of multimeric complexes following receptor-ligand binding is another characteristic of this superfamily. Such complexes, which appear to influence receptor binding affinity, may be hetero-oligomeric, as in the case of interleukin-2 (IL-2),14 IL-3,15 IL-5,16 granulocyte-macrophage colony-stimulating factor,17 and IL-6 for receptors, or homodimeric, as in the case of receptors for growth hormone19 and granulocyte colony-stimulating factor.20 The hydrodynamic behavior of solubilized, native erythropoietin-receptor complexes,21 as well as studies using bifunctional cross-linking agents,22,23 have indicated that this receptor-ligand interaction also involves formation of a multimeric complex, and this has been supported by cotransfection experiments.24 Other studies using a mutated erythropoietin receptor in which arginine 129 in the N-terminal domain was replaced by cysteine have demonstrated that the erythropoietin receptor can form functionally active homodimers.25 Furthermore, the envelope protein of the spleen focus-forming virus (SFFV) interacts directly with membrane-bound erythropoietin receptors to activate them and abrogate the erythropoietin dependence of SFFV-infected erythroid cells,26-28 supporting a role for protein-protein interaction in receptor activation.

Attempts to resolve the nature of the erythropoietin-receptor complex and its signal transduction mechanism have been frustrated by the low number of receptors expressed by normal or transformed erythroid cells.29 Expression of the extracytoplasmic domain of murine30 and human31 erythropoietin receptors has been achieved in mammalian cells. Expression of the extracytoplasmic domain of the human erythropoietin receptor as a fusion protein has also been accomplished in a prokaryotic system,32 but the yield was low due to inclusion-body formation. Attempts to express the full-length receptor in a prokaryotic expression system in our laboratory were also unsuccessful due to the formation of insoluble protein complexes. Baculovirus-infected insect cells provide an alternative heterologous protein expression system that is not only efficient but also has the advantage of being eukaryotic.33 In this report, we describe the isolation and characterization of the full-length murine erythropoietin receptor expressed by Spodoptera frugiperda (Sf9) cells infected with a recombinant baculovirus vector containing cDNA coding for this receptor.

MATERIALS AND METHODS

Reagents. Recombinant human erythropoietin (100,000 U/mg protein) was obtained from the Pharmaceutical Research Institute (Raritan, NJ), and rabbit antiserum to the N-terminal and carboxyl-terminal domains of the murine erythropoietin receptor were from Dr Harvey Lodish (Whitehead Institute, Cambridge, MA). Iodine 125 was purchased from Amersham (Arlington Heights, IL). Tran <sup>35</sup>S-label from ICN Pharmaceuticals Inc (Plainview, NY), Affigel 15
Table 1. Total Cellular Protein Following Infection of Sf9 Cells With Recombinant Baculovirus Containing Erythropoietin Receptor cDNA

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Total Protein (mg/mL)</th>
<th>Cell Viability (%)</th>
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<tbody>
<tr>
<td>0</td>
<td>2.9</td>
<td>90</td>
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<tr>
<td>24</td>
<td>3.9</td>
<td>90</td>
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<tr>
<td>48</td>
<td>4.9</td>
<td>86</td>
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<tr>
<td>72</td>
<td>5.3</td>
<td>64</td>
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Sf9 cells at a concentration of 2 × 10^6/mL were infected with recombinant baculovirus at a multiplicity of infection of 10 in spinner flasks. At indicated time intervals, aliquots of cells were taken for determination of total protein and viability.

from Bio-Rad Laboratories (Richmond, CA), Concanaevalin A (Con A),—Sepharose from Pharmacia (Piscataway, NJ), and wheat germ agglutinin-agarose, a-methyl mannoside, N-acetyl glucosamine, CHAPS, Tween 20, Nonidet P-40, bovine y-globulin, bovine serum albumin (BSA), and polyethylene glycol 6000 (PEG) from Sigma (St Louis, MO). Grace’s complete medium containing 0.33% lactalbumin hydrolysate and 0.33% yeastolate, and fetal bovine serum were from Paragon Biotech Inc (Baltimore, MD). Nitrocellulose paper was purchased from Bio-Rad and PVDF membranes from Millipore Corp (Bedford, MA). Disuccinimidyl suberate (DSS) and iodogen were from Pierce (Rockford, IL). Autographa californica nuclear polyhedrosis virus (AcMNPV) and the baculovirus transfer vector, pVL941, were obtained from Dr M. Summers of Texas A&M University, and cDNA for the murine erythropoietin receptor was obtained from Alan D’Andrea (Children’s Hospital, Boston, MA). 

Cells. Sf9 cells obtained from either the American Type Culture Collection (Rockville, MD) or Paragon were cultured at 27°C in Grace’s complete medium supplemented with 10% heat-inactivated fetal bovine serum and gentamicin (50 μg/mL) either in monolayers or in spinner flasks stirred at 70 to 80 rpm. Cell viability was determined by trypsin blue exclusion. For metabolic labeling, 3 x 10^6 Sf9 cells were incubated in 2 mL methionine-free Grace’s medium at 27°C. After 1 hour, the medium was replaced with an equal volume of methionine-free medium containing 100 μCi/mL Tran 35S-Label and incubation was continued for 3 hours, at which time the cells were washed twice with methionine-free Grace’s medium and harvested by centrifugation at 400 x g at room temperature for polyacrylamide gel electrophoresis and fluorography.

Production of the recombinant baculovirus vector. The cDNA for the murine erythropoietin receptor was cloned into the unique BamHI site of pVL941. Recombinant baculovirus was produced by cotransfecting 2 x 10^6 Sf9 cells with 1 μg AcMNPV DNA and 2 μg of the pVL941 construct using calcium phosphate coprecipitation. Culture supernatants were harvested after 5 to 7 days and screened for homologous recombination by visual inspection of plaques. Recombinants were confirmed by dot-blot hybridization using a 32P-labeled cDNA probe. Purified recombinant baculovirus was obtained by three cycles of plaque purification. For infection of Sf9 cells, viral stocks were generated with a titer of 10^9 plaque-forming units/mL.

Erythropoietin receptor production. For production of erythropoietin receptor protein, Sf9 cells at a concentration of 1 x 10^6 in monolayer culture or 2 x 10^6/mL in suspension culture were infected at a multiplicity of infection of 10 with the recombinant baculovirus. Preliminary time-course studies of total cell protein content and qualitative analysis of protein production using metabolically labeled cells indicated that receptor protein production was maximal 48 hours after infection (Table 1). At this time, aliquots of cells were obtained for counting and assay of cell viability, which was usually at least 90%, and cells were harvested by centrifugation at 400g for 5 minutes at room temperature. The cell pellet was resuspended at a concentration of 10^7 cells/mL in ice-cold lysis buffer (10 mmol/L sodium phosphate, pH 7.4, containing 10% glycerol, 0.6% CHAPS, 0.5 mmol/L dithiothreitol [DTT], 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], 5 μmol/L aprotinin, 8 μmol/L pepstatin, and 2 μg/mL leupeptin) and rocked for 1 hour at 4°C. The suspension was centrifuged at 4°C for 15 minutes at 10,000g, and the pellet was discarded. The receptor could also be solubilized in a biologically active form using Triton X-100, but better yields were obtained with CHAPS (data not shown) and the amount of detergent could be reduced to 0.03% without affecting receptor solubility.

Examination of concentrated culture supernatants by polyacrylamide gel electrophoresis and Western blotting failed to reveal any soluble receptor protein (data not shown).

Chromatography of the solubilized erythropoietin receptor. For affinity chromatography using Con A—Sepharose, the beads were equilibrated with 10 mmol/L Tris hydrochloride, pH 7.5, and 150 mmol/L NaCl (TBS) containing 1 mmol/L CaCl2 and 1 mmol/L MnCl2. Two milliliters of whole-cell lysate adjusted to a concentration of 1.5 mg/mL with lysis buffer was precleared with 100 μL packed glycine-blocked Sepharose beads and then mixed with 100 μL packed Con A—Sepharose beads at 4°C for 12 hours. The beads were washed five times with 1 mL TBS, and adherent proteins were eluted with 0.5 mmol/L α-methyl mannoside in TBS containing 1 mmol/L DTT, 0.5 mmol/L EDTA, 0.06% CHAPS, and 1 mmol/L PMSF. For binding studies with wheat germ agglutinin-agarose, the derivatized beads were equilibrated with 150 mmol/L NaCl and 50 mmol/L HEPES, pH 7.5. One milligram of lysate protein in 1 mL lysis buffer precleared with glycine-blocked Sepharose beads was mixed with 200 μL derivatized packed beads at 4°C for 12 hours. The beads were washed five times with 1 mL 150-mmol/L NaCl and 50 mmol/L HEPES, pH 7.5, and adherent proteins were eluted with 0.3 mmol/L N-acetyl glucosamine in 150 mmol/L NaCl and 50 mmol/L HEPES, pH 7.5, containing 1 mmol/L DTT, 0.06% CHAPS, and 1 mmol/L PMSF. Glycerol was added to each eluate to a final concentration of 10%.

For affinity chromatography with immobilized erythropoietin, recombinant human erythropoietin (1 mg/mL gel) was coupled to Affigel-15 according to the manufacturer’s instructions. Coupling efficiency was 60% as measured by absorbance at 280 nm or by using a trace amount of 125I-labeled erythropoietin. For binding studies, either 100 μg lysate protein in 40 μL lysis buffer or 100 μg Con A eluate in 300 μL TBS was mixed with 250 μL packed erythropoietin-agarose beads for 12 hours at 4°C. The beads were washed five times with 1 mL 100-mmol/mL MOPS, pH 7.5, and adherent protein was eluted by exposure to 0.5 mmol/mL KSCN for 2 hours.

Cell fractionation. To examine the distribution of erythropoietin receptor protein in infected Sf9 cells, the cells were fractionated into cytosolic and membrane components using the technique of Lin et al. Although it was designed for mammalian cells, Sf9 cells behaved comparably. Forty-eight hours after infection, Sf9 cells were washed, collected by centrifugation, suspended in 10 vol (wt/vol) 50-mmol/L mannitol and 5-mmol/L HEPES, pH 7.4, and homogenized by repeated passage through a 25-gauge needle. CaCl2 was added to a final concentration of 10 mmol/L, and after vortexing vigorously for 1 minute, the mixture was centrifuged for 1 minute at 15,600g. The supernatant was centrifuged at 430,000g in a Beckman TL-100 ultracentrifuge (Fullerton, CA) for 6 minutes, and the pellet containing plasma membranes was resuspended in 50 mmol/L Tris hydrochloride and 1 mmol/L EDTA, pH 8.0. To validate this procedure for Sf9 cells, infected and noninfected Sf9 cells were incubated with 32P-erythropoietin for 3 hours in the presence or absence of NaN3 and then exposed to DSS to cross-link ligand bound to the cell surface as described below. After cross-linking, cells
were counted in a gamma scintillation counter and then plasma membranes were prepared as described earlier and subjected to scintillation counting. Recovery of $^{125}$I-erythropoietin on noninfected cell plasma membranes was negligible (0.6%), whereas 83% of the ligand was recovered with plasma membranes of infected SF9 cells exposed to Na$_2$N. Only 29% of the ligand was recovered with plasma membranes of cells not exposed to Na$_2$N.

Western blotting of erythropoietin-receptor protein. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of protein samples was performed according to the technique of Laemmli. For Western blotting, protein was transferred from the gel to nitrocellulose paper using a Bio-Rad Transblot apparatus set at 20V for 12 hours at 4°C. Fast-green staining was used to confirm adequacy of the transfer, and then the blots were probed with the appropriate antiserum. Antibody binding was detected calorimetrically using goat-antirabbit antiserum coupled to alkaline phosphatase (Promega, Madison, WI) according to the manufacturer’s instructions.

Ligand blotting of the erythropoietin-receptor protein. Receptor protein was transferred from a polyacrylamide gel to a PVDF membrane at 23°C for 30 minutes using a Bio-Rad semidry-transfer cell set at 20V. Denatured proteins were renatured according to the technique of Ferrell and Martin. Briefly, the membrane was soaked at 4°C in 7 mol/L guanidine hydrochloride, 50 mmol/L Tris hydrochloride, 50 mmol/L DTT, and 2 mmol/L EDTA, pH 8.3, for 1 hour and incubated overnight at 4°C in 100 mmol/L NaCl, 50 mmol/L Tris hydrochloride, 2 mmol/L EDTA, 2 mmol/L DTT, 0.1% (wt/vol) Nonidet P-40, and 1% (wt/vol) BSA, pH 7.5. The membrane was washed with 1% BSA in 10 mmol/L Tris hydrochloride, 150 mmol/L NaCl, pH 8.0, and 1% Tween 20 and exposed to $^{125}$I-erythropoietin for 3 hours at room temperature. The membrane was then washed with 10 mmol/L Tris hydrochloride, pH 8.0, 150 mmol/L NaCl, and 1% Tween 20, and autoradiography was performed at -80°C using Kodak XAR film (Eastman Kodak, Rochester, NY).

Ligand binding studies. Ligand binding studies were performed on whole cells or whole-cell lysates. For whole-cell–ligand equilibrium binding studies, preliminary studies indicated that ligand binding was more satisfactory when SF9 cells were attached to a surface on whole cells or whole-cell lysates. For whole-cell-ligand equilibrium binding studies, preliminary studies indicated that ligand binding was more satisfactory when SF9 cells were attached to a surface. For determination of nonspecific binding, a 40- to 100-fold excess of unlabeled erythropoietin was added simultaneously to selected ligand-labeled protein complexes. Western blotting of protein-renaturation assay. Whole-cell lysates following polyacrylamide gel electrophoresis revealed nine proteins for which synthesis was increased in infected cells as compared with noninfected cells. Of particular interest was a group of proteins with molecular masses between 60 and 66 kD for which synthesis was maximal at 48 hours (Fig 1).

RESULTS

Erythropoietin receptor synthesis by SF9 cells. The kinetics of protein synthesis in SF9 cells infected with recombinant baculovirus containing the erythropoietin receptor cDNA were analyzed by metabolic labeling for 3 hours with a mixture of $^{35}$S-methionine and $^{35}$S-cysteine at 24, 48, and 72 hours after infection. Fluorography of solubilized whole-cell lysates following polyacrylamide gel electrophoresis revealed nine proteins for which synthesis was increased in infected cells as compared with noninfected cells. Of particular interest was a group of proteins with molecular masses between 60 and 66 kD for which synthesis was maximal at 48 hours (Fig 1).

Both immunologic and functional assays were used to determine whether the erythropoietin receptor was represented among this group of proteins. Western blotting of whole-cell lysates from SF9 cells harvested 48 hours after infection using polyclonal antisera reactive with either the amino- or carboxyl-terminus of the murine erythropoietin receptor revealed the presence of immunologically reactive proteins with molecular masses ranging from 62 to 76 kD that were not present in uninfected SF9 cells (Fig 2). Additionally, a doublet of 39 to 37 kD was also present only in infected SF9 cells that shared immunologic identity with the erythropoietin receptor. A similar pair of proteins has been observed in Ba/F3 and FCP-1 following transfection with murine erythropoietin receptor cDNA and is probably a consequence of proteolytic degradation.

The functional behavior of these proteins was analyzed by ligand blotting using a protein-renaturation assay. Whole-cell lysates from SF9 cells harvested 48 hours after infection were subjected to polyacrylamide gel electrophoresis. A prominent band with a molecular mass of approximately 62 kD was present in infected SF9 cell lysates as compared with...
ISOLATION OF THE ERYTHROPOIETIN RECEPTOR

123

P

200

116-

97-

66-

45-

Autoradiography of the exposed membrane (Fig 3B) revealed a single band with a molecular mass of 62 kD, which was absent in uninfected Sf9 cell lysates. Interestingly, binding of erythropoietin to receptors immobilized on a PVDF membrane was so strong it could not be completely away (data not shown).

The functional behavior of the erythropoietin receptor expressed in Sf9 cells was also confirmed by binding of the receptor to recombinant human erythropoietin immobilized on Affigel-15, as shown in Fig 4.

 Binding of 125I-erythropoietin by the erythropoietin receptor expressed in Sf9 cells. Sf9 cells infected with recombinant baculovirus containing cDNA for the full-length murine erythropoietin receptor inserted the receptor in a functional form and in the correct orientation in the plasma membrane. Intact infected Sf9 cells actively internalized 125I-erythropoietin (Fig 5A). However, in contrast to erythroid progenitor cells, receptor internalization following ligand binding was much slower. Figure 5B illustrates a typical equilibrium binding isotherm for Sf9 cells expressing full-length erythropoietin receptors 48 hours after infection. Scatchard analysis (Fig 5C) revealed a single binding affinity (kD, 330 pmol/L; range, 20 to 760). The wide variation in kD appeared to be a consequence of the number of surface receptors expressed, since, as shown in Fig 5D and confirmed by linear regression analysis, there was a strong inverse correlation (r = .90 and P < .001) between receptor kD and receptor copy number (range, 6 x 10^3 to 8 x 10^4/cell).

uninfected cells (Fig 3A). Following transfer to a PVDF membrane, proteins were denatured in 7 mol/L guanidine hydrochloride and renatured overnight in 0.1% Nonidet P-40. The PVDF membrane was then exposed to 125I-labeled recombinant human erythropoietin (80 pmol/L) for 3 hours.

Fig 1. Metabolic labeling of Sf9 cells infected with recombinant baculovirus containing cDNA coding for erythropoietin receptor. Aliquots of Sf9 cells were incubated at 24 (lane 1), 48 (lane 2), and 72 (lane 3) hours after infection in methionine-free medium with 100 μCi/mL Tran 35S-Label for 3 hours before harvesting for SDS-7.5% polyacrylamide gel electrophoresis and fluorography. Uninfected Sf9 cells (lane 4) were treated in the same fashion after 72 hours of culture.

Fig 2. Western analysis of solubilized whole-cell lysates from non-infected (lanes 1, 3, 5, and 7) and infected (lanes 2, 4, 6, and 8) Sf9 cells harvested after 48 hours. The nitrocellulose membrane was probed with a polyclonal antiserum to the carboxyl-terminus of the erythropoietin receptor (lanes 1 and 2), a polyclonal antiserum to the amino-terminus (lanes 3 and 4), normal rabbit serum (lanes 5 and 6), and amino-terminus antiserum after incubation with a synthetic peptide corresponding to the first 14 amino acids of the amino-terminus (lanes 7 and 8).
Based on the behavior of the solubilized receptor described above, it was possible to purify it by sequential affinity chromatography on Con A-Sepharose and erythropoietin-agarose as shown in Fig 8. Since there is no specific assay for determining receptor specific activity, the extent of purification and the yield through the Con A step cannot be quantified, but starting with 9 mg solubilized whole-cell lysate, 90 μg partially purified receptor protein could be recovered.

Distribution of the erythropoietin receptor in Sf9 cells. Since a smaller proportion of the receptor bound to wheat

Isolated plasma membranes and whole lysates of infected Sf9 cells also bound erythropoietin with a single affinity (Fig. 6), but in contrast to cell-surface receptors for erythropoietin, the kD was much higher, 57 and 92 pmol/L, respectively. In a few experiments in which very high numbers of receptors were generated, low-affinity binding (172 nmol/L) was also observed with whole-cell lysates (data not shown). Whether this reflects the immature nature of these receptors is unknown, since pulse-chase studies to examine receptor processing were not performed.

Affinity chromatography of the solubilized erythropoietin receptor. Since the erythropoietin receptor is normally glycosylated, it was of interest to determine its glycosylation state during expression in Sf9 cells. The erythropoietin receptor produced in Sf9 cells bound to both immobilized Con A and wheat germ agglutinin (Fig 7A) and retained biologic activity after elution from the affinity resins (Fig 7B).
Fig 5. (A) Binding and endocytosis of $^{125}$I-erythropoietin by infected Sf9 cells. Forty-eight hours after infection, Sf9 cells were incubated with $^{125}$I-erythropoietin at 27°C in the presence or absence of 0.02% sodium azide or excess unlabeled erythropoietin. At indicated time intervals, cells were washed and harvested for quantitation of $^{125}$I-erythropoietin uptake. (C) Total $^{125}$I-erythropoietin uptake; (Δ) total binding in the presence of sodium azide; (△) nonspecific binding. (B) Equilibrium binding of $^{125}$I-erythropoietin to intact Sf9 cells. Forty-eight hours after infection, Sf9 cells were exposed to varying concentrations of $^{125}$I-erythropoietin in the presence or absence of excess unlabeled erythropoietin for 5 hours at 4°C before harvesting. (C) Total $^{125}$I-erythropoietin binding; (Θ) specific binding; (Δ) nonspecific binding. SD for each data point was less than 10%. (D) Scatchard analysis of the data in B. (D) Plot of receptor $K_d$ vs receptor number for intact Sf9 cells exposed to $^{125}$I-erythropoietin 48 hours after infection (regression equation, $y = 9.4 \times 10^{-12}x + 2.2 \times 10^{-8}$, $r = .80$ and $P = < .001$).

germ-agarose as compared with Con A-Sepharose, it seemed likely that this receptor represented a more mature protein. Accordingly, we analyzed distribution of the receptor with respect to glycosylation state in Sf9 cells 48 hours after infection. For this purpose, infected cells were first separated into cytosol and plasma membrane fractions, and solubilized proteins from these fractions were chromatographed on either Con A-Sepharose or wheat germ-agarose and analyzed by polyacrylamide gel electrophoresis and Western blotting. Membrane-bound receptors had a greater apparent molecular mass than cytosolic receptors, and the bulk of receptors that bound to immobilized wheat germ agglutinin were present in the plasma membrane fraction (Fig 9).

Cross-linking of $^{125}$I-erythropoietin to Sf9 cells expressing the erythropoietin receptor. Using the homobifunctional cross-linker, DSS, labeled erythropoietin was coupled to proteins on the surface of Sf9 cells, and, after cross-linking, migrated with molecular masses of approximately 100 and 125 kD (Fig 10). Similar observations have been made in COS cells transfected with the murine erythropoietin receptor. An identical result was obtained with the water-soluble
cross-linker, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide ([EDAC] data not shown), and when cross-linking was performed using solubilized whole-cell lysates (Fig 11).

To determine the identity of the two proteins cross-linked to erythropoietin, cross-linked complexes from whole-cell lysates were immunoprecipitated using antibodies to the carboxyl-terminal portion of the erythropoietin receptor and analyzed by polyacrylamide gel electrophoresis. Both the 100- and 125-kD complexes were immunoprecipitated, indicating that each complex contained erythropoietin receptor protein (Fig 11). Similar results were obtained with an N-terminal antiserum (data not shown).

**DISCUSSION**

Sf9 cells infected with a recombinant baculovirus containing cDNA coding for the full-length murine erythropoietin receptor produced large quantities of receptor protein that could be solubilized using CHAPS in a form capable of binding erythropoietin. The erythropoietin receptor synthesized by these cells was glycosylated before insertion in the
correct orientation into the plasma membrane, and underwent endocytosis after binding to its ligand. Scatchard analysis of binding isotherms using 125I-erythropoietin revealed that erythropoietin receptors expressed on the surface of infected Sf9 cells bound the hormone with a single affinity and a kD 330 pmol/L. Solubilized receptors in whole-cell lysates and receptors in plasma membranes isolated from infected Sf9 cells exhibited higher-affinity binding (92 and 57 pmol/L, respectively) than cell-surface receptors. Whether the difference in assay methodologies could account for this is unknown. Binding affinities of membrane-bound and solubilized receptors were higher than found by Harris et al[12] for the extracytoplasmic domain of the human erythropoietin receptor immobilized as a fusion protein on glutathione-agarose (1.5 nmol/L), and by Yet and Jones[7] for a soluble form of the extracytoplasmic domain of the human erythropoietin receptor (1.1 nmol/L). By contrast, COS cells transiently transfected with a plasmid containing the same cDNA used in our studies expressed erythropoietin receptors exhibiting two different binding affinities for erythropoietin, with kD values of 30 and 210 pmol/L, respectively[6].

With respect to erythropoietin receptors expressed on the surface of Sf9 cells, binding affinity varied inversely with receptor copy number. This could be due to steric hindrance or production of dysfunctional receptors. Alternatively, this could be a reflection of differences in the milieu of insect and mammalian plasma cell membranes. The actual difference in receptor copy number between experiments was probably due to differences in the efficiency of infection in different batches of cells and the timing of cell harvests, since the same lot of recombinant baculovirus was used for all infections for the data shown. However, the inverse reciprocal relationship between receptor copy number and affinity was not restricted to a single virus lot. Although erythropoietin receptors in intact Sf9 cells, Sf9 plasma membranes, or whole-cell lysates exhibited only a single binding affinity, receptor-ligand cross-linking with DSS yielded results similar to those obtained with COS cells transfected with erythropoietin receptor cDNA[5]. In both situations, erythropoietin was cross-linked to proteins with molecular masses of 90 to 105 and 65 kD. By immunoprecipitation (Fig 11), we demonstrated that both of these proteins in Sf9 whole-cell lysates were recognized by erythropoietin receptor antibodies and thus presumably represent differentially modified erythropoietin receptor proteins. It is unlikely that either represents nonreceptor protein, since similar results were obtained by cross-linking with solubilized receptors and membrane-bound receptors and when EDAC was used in place of DSS[23] (data not shown). In
Fig 8. Purification of the full-length murine erythropoietin receptor from solubilized whole-cell lysates of infected SF9 cells. Whole-cell lysates from infected (lanes 1, 3, and 5) and noninfected (lanes 2, 4, and 6) cells were sequentially chromatographed on Con A-Sepharose (lanes 2 and 4) and erythropoietin-agarose beads (lanes 5 and 6), and eluted proteins were analyzed by SDS-10% polyacrylamide gel electrophoresis with Coomassie blue staining.

SF9 cells the most abundant species was the 65-kD protein, whereas in COS cells the 90-kD protein was more abundant. However, the identity of proteins cross-linked to erythropoietin in COS cells has not been established. Finally, in experiments not reported here, $^{125}$I-erythropoietin incubated with erythropoietin-dependent, murine HCD-57 erythroleukemia cells could be cross-linked with DSS to proteins of 90 and 110 kD. The relation of these proteins to those cross-linked to erythropoietin on SF9 cells is also unknown.

That there was heterogeneity with respect to receptor molecular weight was apparent from immunoblotting, ligand blotting, and affinity chromatography of the receptors on immobilized erythropoietin. Thus, although immunoblotting revealed a broad band of identity (Fig 2), with ligand blotting, only a narrow band of binding protein was apparent with a molecular mass of 62 kD (Fig 3B). Similarly, receptor protein that bound to erythropoietin-agarose also exhibited a restricted mobility on polyacrylamide gel electrophoresis with an apparent molecular mass of 62 kD (Fig 4). This suggests that of the erythropoietin receptors produced in SF9 cells, the most abundant species was most prominent in the ligand binding assays. Similar observations have been made by Atkins et al using mammalian erythroid cell membranes.

Although spontaneous homodimerization of the murine erythropoietin receptor has been observed with mutant receptors bearing a cysteine at residue 129 of the extracytoplasmic domain, binding of erythropoietin to a soluble construct of the extracytoplasmic domain failed to induce oligomerization. The hydrodynamic characteristics of solubilized receptor-erythropoietin complexes from splenic membranes of mice infected with the anemia strain of the Friend virus also suggested the existence of erythropoietin-receptor...
Fig 10. Cross-linking of \( ^{125}\text{I}\)-erythropoietin to surface proteins of intact infected SF9 cells. SF9 cells were incubated at 4°C for 5 hours with 7.6 ng \( ^{125}\text{I}\)-erythropoietin in the absence (lane 1) or presence (lane 2) of 850 ng unlabeled erythropoietin before cross-linking with DSS (lanes 1 and 2). Lane 3, infected SF9 cells exposed to \( ^{125}\text{I}\)-erythropoietin without cross-linking.

multimers, but cross-linking studies failed to confirm this.\(^{21}\) Similarly, cross-linking of erythropoietin bound to infected SF9 cells or solubilized whole-cell lysates failed to reveal multimeric receptor-ligand complexes. Thus, the physiologic correlates required for erythropoietin-receptor oligomerization remain to be established. However, taking everything together, the baculovirus expression system using SF9 cells provides a means of obtaining large quantities of full-length erythropoietin receptor protein that is easily solubilized and purified in a form that binds erythropoietin. Additionally, recombinant baculovirus–infected SF9 cells provide a model system to analyze in a eukaryotic cell the synthesis, processing, and turnover following ligand binding of the full-length erythropoietin receptor, as well as receptor-ligand interactions in solution.

Fig 11. Immunoprecipitation of \( ^{125}\text{I}\)-erythropoietin cross-linked complexes in SF9 whole-cell lysates. Infected and noninfected SF9 whole-cell lysates were incubated with \( ^{125}\text{I}\)-erythropoietin for 24 hours followed by exposure to DSS. Carboxyl-terminal antibodies were used for immunoprecipitation, and immunoprecipitates were harvested with protein A–Sepharose and analyzed by SDS–7.5% polyacrylamide gel electrophoresis. Lane 1, infected SF9 cell lysate immunoprecipitated with carboxyl-terminal antibodies; lane 2, infected SF9 cell lysate immunoprecipitated with preimmune rabbit serum; lane 3, noninfected cell lysate immunoprecipitated with carboxyl-terminal antibodies.
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