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Mutations in the WSAWSE and Cytosolic Domains of the Erythropoietin Receptor Affect Signal Transduction and Ligand Binding and Internalization

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The terminal development of erythroid progenitor cells is promoted in part through the interaction of erythropoietin (EPO) with its cell surface receptor. This receptor and a growing family of related cytokine receptors share homologous extracellular features, including a well-conserved WSXWS motif. To explore the functional significance of this motif in the murine EPO receptor, five WSAWSE mutants were prepared and their signal-transducing, ligand binding, and endocytotic properties were compared. EPO receptors mutated at tryptophan residues (W-232, W-235→G; W-235→G; W-235→F) failed to mediate EPO-induced growth or pp100 phosphorylation, while S-236→T and E-237→K mutants exhibited partial to full activity (50 to 100% of wild-type growth and induced phosphorylation). Ligand affinity was reduced for mutant receptors (two- to fivefold), yet expression at the cell surface for all receptors was nearly equivalent. Also, the ability of mutated receptors to internalize ligand was either markedly reduced or abolished (W-235→F), indicating a role for the WSAWSE region in hormone internalization. Interestingly, receptor forms lacking 97% of the cytosolic domain (no signal-transducing capacity; binding affinity reduced two- to threefold) internalized EPO efficiently. This and all WSAWSE receptor forms studied also mediated specific cross-linking of ^{125}I-EPO to three accessory membrane proteins (M_rs, 120,000, 105,000, and 93,000). These findings suggest that the WSAWSE domain of the EPO receptor is important for EPO-induced signal transduction and ligand internalization. In contrast, although the cytosolic domain is required for growth signaling, it appears nonessential for efficient endocytosis.

Erythropoietin (EPO) is a glycoprotein hormone that stimulates the proliferation and differentiation of erythroid progenitor cells through its interaction with lineage-specific cell surface receptors (26). Receptors for EPO have been identified on erythroid cells and leukemic cell lines (8), and cDNAs encoding murine and human EPO receptors (82% amino acid identity) have been cloned (6, 22, 50). Recent analyses of the cytosolic domains of these receptors through deletional mutation and expression in heterologous cells have led to the identification of several functional subdomains. We have shown previously that the carboxy-terminal region of the EPO receptor is nonessential for proliferative signal transduction in transfected FDC-P1 cells, with limited activity retained by mutants possessing only 81 membrane-proximal cytosolic residues (of 236 total) (39). Studies by others suggest that a 40-amino-acid carboxy-terminal domain negatively regulates proliferative signaling (7), possibly through the tyrosine phosphorylation of this region (53). In addition, two cytosolic domains essential for both EPO-induced tyrosine phosphorylation and growth have been identified: an 80- to 90-amino-acid membrane-proximal domain and a 20-residue region that shares recognizable homology with the interleukin 2 (IL-2) receptor beta chain (5, 7, 33).

While no enzymatic activities are encoded by the EPO receptor (or related cytokine receptors) (1, 4), we have also demonstrated EPO-induced tyrosine phosphorylation in responsive B6SUt.EP cells (41) and in myeloid FDC-P1 cells stably expressing the murine EPO receptor (i.e., FDC-ER cells) (40). In FDC-ER cells, tyrosine phosphorylation of cytosolic protein with an M_r of 100,000 (pp100) is induced by EPO, IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition, the cytosolic domain of the EPO receptor was shown to be required for this activity. These results support and extend previous studies which have suggested overlap in the proliferative signal transduction pathways activated by these related receptor systems (2, 23, 29, 33).

Advances in the cloning and analysis of cytokine receptors have led to the identification of a superfamily of hematopoietic receptors which includes the EPO receptor (1, 4), the receptors for IL-2 (beta chain), IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, G-CSF, growth hormone, and prolactin, and, most recently, the receptors for ciliary neutrophic factor (9) and leukemia inhibitory factor (15). These receptors are characterized by common extracellular features, including the conservation of four periodically spaced cysteines and a WSXWS motif (W, tryptophan; S, serine; X, a nonconserved amino acid). While the precise role of the WSXWS sequence is not known, it has been suggested that this sequence is essential for ligand binding and growth signaling in the G-CSF and IL-2 receptor systems (13, 34). This is consistent with computer-generated structural models which depict the WSXWS motif helping to form the floor of a binding crevice (1). Notably, the growth hormone receptor lacks a conserved WSXWS motif (Tyr-Gly-Glu-Phe-Ser), and crystallography has shown that this region is located away from the ligand-binding interface (10). Interestingly, the WSXWS sequence also has been identified in the IL-6 receptor-associated protein and oncostatin M receptor,

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gp130 (14, 36), as well as in the non-ligand binding subunits of the human and murine GM-CSF receptors (20, 24).

In order to investigate the functional significance of the WSAWSE motif and to further study the role of the cytosolic domain of the EPO receptor, we have constructed various WSAWSE mutants, as well as a receptor form essentially lacking the cytosolic domain. Through the expression of these constructs in myeloid FDC-P1 cells, we have compared receptor biosynthetic processing, binding and endocytotic properties, and the ability of each receptor to mediate EPO-induced growth. In addition, the capacity of mutated receptors to mediate EPO-induced tyrosine phosphorylation of pp100 and to specifically cross-link receptor-associated membrane proteins was analyzed.

MATERIALS AND METHODS

Cell culture, growth factors, and proliferation assays. Murine FDC-P1 cells (11), a myeloid cell line normally dependent on IL-3 or GM-CSF for growth, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.01 mM 2-mercaptoethanol, and 2.5% conditioned medium from WEHI-3 cells. COS cells (American Type Culture Collection) and the murine erythroleukemia cell line, J2E-1 (25), were grown in this medium lacking WEHI-3-conditioned medium. The purified growth factors used were recombinant human EPO (150,000 U/mg) (R. W. Johnson Pharmaceutical Research Laboratories, La Jolla, Calif.), recombinant murine GM-CSF (Immunex, Seattle, Wash.), and synthetic murine IL-3 (Biomedical Research Center, Vancouver, British Columbia, Canada). Proliferation assays were performed with [^{3}H]thymidine as described elsewhere (39).

EPO receptor cDNA constructs. The plasmids used in these studies were prepared from the murine EPO receptor cDNA clone 190 (6) in the expression vector pXM (51). In the construction of pXMBS, the EPO receptor cDNA was cloned into pBlueScribe II K/S+ (pBKS-ER) and restricted with *Bgl*II, and a 14-bp *Xba*I stop linker (Pharmacia) was inserted at this blunted site. The altered construct then was cloned into pXM. Site-directed mutagenesis of the WSAWSE region was accomplished by a T7-GEN in vitro mutagenesis strategy (U.S. Biochemical). Briefly, mutant single-stranded oligonucleotides were annealed to single-stranded template DNA (pBKS-ER), extended in the presence of 5-*methyl*-dCTP, and ligated to produce a heteroduplex of DNA. The parental strand was then nicked with the restriction enzymes *Msp*I, *Sau*3AI, and/or *Hha*I and removed by digestion with exonuclease III. Methylated single-stranded molecules were transformed into a mutant *mcrAB* strain of *Escherichia coli*, and mutants were identified by sequencing.

Gene transfer. EPO receptor cDNA constructs and pSV2-neo were stably transfected into FDC-P1 cells by electroporation and selected in G418 (1 mg/ml) as described elsewhere (39). Sublines were established by dilutional cloning and were maintained in DMEM containing WEHI-3-conditioned medium (see above). Transient expression of pXMBS in COS cells was accomplished by using a published lipofection procedure (12).

Endo H and Western analyses. Cell membranes (lacking mitochondrial membranes) were prepared with sucrose step gradients as described previously (41) and were suspended in 50 mM sodium phosphate (pH 6.0) at a protein concentration of ≤2.0 mg/ml. Sodium dodecyl sulfate (SDS) was added (1.2 mg/mg of membrane protein), and samples were boiled for 15 min. The SDS concentration was reduced to ≤0.2% by the addition of sodium phosphate buffer, and each sample was incubated in the presence (50 mU/ml) or absence of endoglycosidase H (Genzyme; endo H) for 20 h at 37°C. The reactions were terminated by the addition of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, the samples were boiled for 15 min, and equivalent amounts of membrane protein (0.18 mg per lane) were analyzed by SDS-PAGE (27). EPO receptor forms were detected by Western blotting (47) with antisera in rabbits prepared against synthetic peptides with sequences corresponding to those of the predicted amino-terminal and carboxy-terminal domains of the murine EPO receptor (28).

Internalization assays. In receptor-ligand internalization assays, exponentially growing cells (6×10^5 to 8×10^5 cells per ml) were washed three times in phosphate-buffered saline and resuspended in binding buffer (DMEM, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4], 0.2% bovine serum albumin [BSA]) at 1.5×10^7 cells per ml. Cells (0.1 ml) were preincubated at 37°C with 7.5% CO_2 for 30 to 60 min in 96-well plates and then incubated with 1.3 nM ^{125}I-EPO (Amersham; 1,710 Ci/mmol) for 40 min under the conditions described above. To remove unbound ^{125}I-EPO, the cells were layered onto and spun through a 10% sucrose solution in 100 mM NaCl–25 mM HEPES–5 mM EDTA (pH 7.4). Specific internalization was demonstrated both by competition with 200-fold excess unlabeled hormone and by removal of surface-bound ^{125}I-EPO (following the sucrose spin) by incubation with 1 ml of 0.5 M NaCl–0.25 M acetic acid (pH 2.5) for 3 min on ice (44). Pellets were counted in a Beckman 7000 gamma counter.

EPO receptor cross-linking studies. Isolated membranes (see above) were suspended in buffer B (25 mM HEPES [pH 7.4], 1 mM EDTA, 500 ng of leupeptin, 700 ng of pepstatin, and 0.05 mg of phenylmethylsulfonyl fluoride per ml) containing 10% glycerol and were stored at −70°C at a protein concentration of ≥2.0 mg/ml. Prior to cross-linking, the membranes were washed three times with buffer B (Eppendorf Microfuge; 30 min at 16,000 × *g*; 4°C) and resuspended at 2 mg/ml in this buffer. Samples (0.2 ml) were then incubated at 23°C for 2 to 3 h with ^{125}I-EPO (Amersham; 4,000 Ci/mmol) in the presence or absence of 200-fold excess unlabeled EPO. Unbound hormone was removed by washing membranes three times in buffer B, as described above. Pellets were resuspended in buffer B and incubated with 0.2 mM disuccinimidyl suberate (Pierce) at 4°C for 30 min. The reaction was quenched by the addition of an equal volume 0.5 M Tris-HCl–0.5 M glycine (pH 8.0), and the membranes were then washed twice in 10 mM Tris-HCl–1 mM EDTA (pH 8.0). The pellets were then solubilized in SDS-PAGE sample buffer, boiled for 15 min, and analyzed by SDS-PAGE (7.5% gel) and autoradiography.

EPO receptor-binding analyses on whole cells and membrane fractions. Samples were incubated in binding buffer (see above) with 6 nM ^{125}I-EPO (157 Ci/mmol) for single-point analyses or with radiolabeled EPO (1,710 Ci/mmol) at concentrations ranging from 50 to 4,000 pM for Scatchard analyses. Cells (1.5×10^6, 0.15 ml) were incubated with hormone for 18 h at 4°C, and membranes (0.2 mg of membrane protein, 0.1 ml) were incubated for 3 h at 37°C. The samples were sedimented through 10% sucrose (see internalization assays), and associated counts were measured. In parallel samples, nonspecific binding was assayed either with a 100- to 200-fold excess of unlabeled EPO or by subtraction of nonspecific counts associated with parent FDC-P1 cells.

EPO-induced protein tyrosine phosphorylation. In phosphorylation assays, cells were washed free of growth factors and were preincubated at 6×10^5 cells per ml for 14 h in DMEM–1% FBS. Subsequently, the cells were labeled for 2 h in phosphate-free DMEM–1% FBS containing 0.5 mCi of [^{32}P]orthophosphate per ml. Labeled cells were collected and incubated at 2×10^6 to 3×10^6 cells per ml in phosphate-free DMEM–1% FBS–50 μM Na_3VO_4 for 30 min. The cells were then exposed to EPO (10 U/ml) for 30 min, collected by centrifugation (800 × *g* for 2 min), and frozen in a dry ice bath. The length of exposure to Na_3VO_4 was constant for all samples, including controls. Frozen cells were lysed at 4°C in a solution containing 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM Na_3VO_4, and 0.1% BSA, 500 ng of leupeptin, 700 ng of pepstatin, and 50 μg of phenylmethylsulfonyl fluoride per ml, and 10 mM Tris (pH 7.6), and phosphotyrosine-containing proteins were immunoprecipitated with antibodies to phosphotyrosine (PY20; ICN Biochemicals) plus protein G-Sepharose (Pharmacia). [^{32}P]phosphotyrosine-containing proteins were then eluted in sample buffer and analyzed by SDS-PAGE (7% gel) and autoradiography.

RESULTS

WSAWSE and cytosolic domains are necessary for EPO-mediated signal transduction. In previous experiments, we have shown that murine FDC-P1 cells stably expressing a full-length EPO receptor cDNA (i.e., FDC-ER cells) proliferate in response to EPO (39). Also, as many as 155 (of 236 total) carboxy-terminal amino acids could be removed from the cytosolic domain of this receptor without abolishing the ability of these receptors to mediate EPO-induced proliferation. To further delineate functional domains of the murine EPO receptor, receptors were constructed which were either (i) mutated within the conserved WSAWSE region or (ii) deletionally mutated at the cytosolic domain (229 of 236 carboxy-terminal cytosolic residues removed). Each receptor form was then expressed in FDC-P1 cells (Fig. 1), and initially, the ability of each mutant to mediate EPO-induced growth signaling and tyrosine phosphorylation of pp100 was assessed.

The activities of wild-type and mutated EPO receptors in EPO-induced cell proliferation assays are compared in Fig. 2. Receptors mutated at one or both tryptophan residues (W-232 and W-235 [FDC-WG, FDC-2WG, and FDC-2WF cells]) were essentially inactive, as was the cytosolic deletion mutant pXMBS (FDC-BS cells). In contrast, partial to full EPO responsiveness was retained for receptors mutated at serine (S-236→T) or glutamic acid (E-237→K) residues within the WSAWSE domain (FDC-2ST and FDC-EK cells; 50 to 100% responsiveness, respectively). Thus, initial experiments suggested a role for the WSAWSE motif, particularly for each tryptophan residue, in mediating efficient EPO-induced proliferation.

The ability of WSAWSE receptor mutants to mediate EPO-induced phosphorylation of a cytosolic phosphoprotein with an M_r of 100,000 (pp100), as assessed by immunoprecipitation with antiphosphotyrosine antibodies, was also analyzed (Fig. 3). Induced phosphorylation of pp100 was observed only for cells expressing receptor forms that mediated EPO-induced growth (i.e., FDC-ER, FDC-2ST, and FDC-EK cells), while no detectable levels of pp100 phosphorylation were induced by EPO in nonresponsive FDC-WG, FDC-2WG, and FDC-2WF cells. Recently, we have also shown that nonresponsive FDC-BS cells likewise fail to mediate EPO-induced pp100 phosphorylation (40). Thus, intact WSAWSE and cytosolic domains of the murine EPO receptor apparently are necessary for induced pp100 phosphorylation. Since induced phosphorylation was detectable only for receptor forms which are active in growth signaling, a role for pp100 in EPO receptor-mediated proliferative signal transduction is suggested.

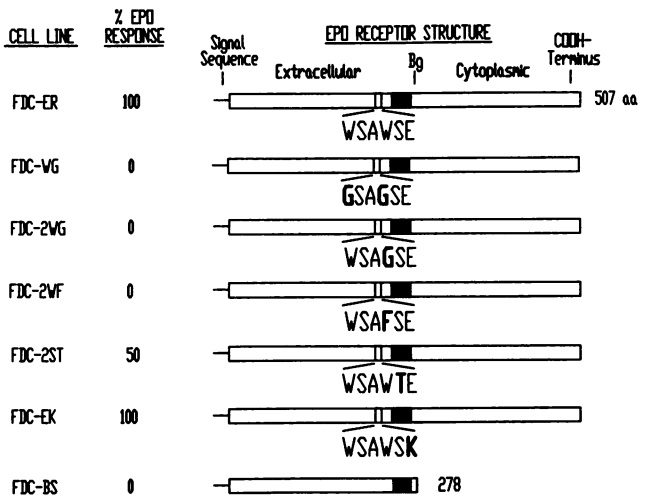

FIG. 1. EPO receptor constructs and derived cell lines. Structures of wild-type and mutated EPO receptors as predicted from cDNA constructs are diagrammed, including the positions of the predicted transmembrane domain (filled boxes), WSAWSE point mutations (boldface type), and *Bgl*II restriction site (Bg) and the lengths of the altered receptors. Also indicated are designations of FDC-P1-derived cell lines expressing these various receptor forms, together with their relative abilities to proliferate in response to maximal doses of EPO (6 U/ml) (% EPO response [FDC-ER = 100%]). aa, amino acid.

Binding properties and biosynthesis of WSAWSE and cyto-

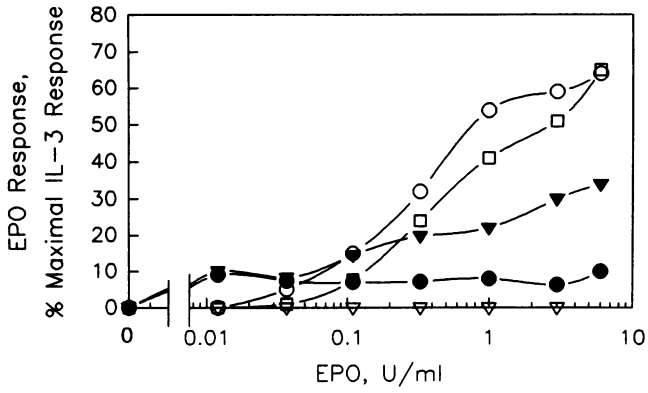

FIG. 2. EPO responsiveness of FDC-P1 cells expressing cytoplasmic deletion and WSAWSE mutant receptors. Rates of EPO-induced [^{3}H]thymidine incorporation were assayed and were normalized by direct comparison with maximal growth rates induced by IL-3 (4 nM). EPO dose-response curves are shown for the following clonal sublines: FDC-ER.5 (open circles); FDC-WG.2, FDC-2WG.36, and FDC-BS.8 (open triangles); FDC-2WF.22 (closed circles); FDC-2ST.49 (closed triangles); and FDC-EK.6 (open squares). Cell lines are as designated in Fig. 1, with subclones indicated by decimal points and numbers. The dose-response curves shown are representative of 4 to 12 subclones tested per cell type.

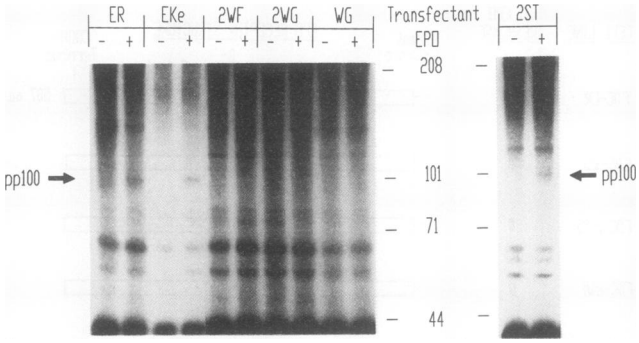

FIG. 3. EPO-induced tyrosine phosphorylation of pp100. FDC-P1 cells expressing wild-type (ER) or WSAWSE mutant receptors were labeled with ^{32}P and exposed to no factor (−) or to 10 U of EPO (+) per ml for 30 min. Phosphotyrosine-containing proteins were then immunoprecipitated from cell lysates with monoclonal antibody PY20, as described in Materials and Methods. Cell lines and subclones are as designated in Fig. 1 and 2, with the exception of FDC-EK cells maintained in EPO (EKe). Molecular size markers (in thousands) and pp100 (arrows) are indicated.

solic receptor mutants. Differential activities of the EPO receptor mutants described above might be explained, in part, by altered ligand binding, receptor biosynthesis, and/or reduced receptor transport to the cell surface. However, analyses of single-point binding on intact cells demonstrated the ability of all receptors to bind hormone and showed approximately equivalent numbers of receptor sites per cell among wild-type and mutant receptors (Table 1). No specific binding of ^{125}I-EPO was detected with control FDC-P1 cells. Equilibrium binding analyses of site affinities for wild-type versus mutant receptors also were performed (Table 2). Among WSAWSE receptor mutants, cells expressing WG, 2WF, 2ST, and EK receptors showed only a two- to fivefold decrease in binding affinity, despite marked differences in the ability of those receptors to mediate EPO-induced mitogenic signaling (Fig. 2; see above). On the basis of analyses with both whole cells and isolated membranes, truncated BS receptors showed an affinity for EPO (K_d = 1.4 nM) that was two- to threefold less than that of wild-type receptors (K_d = 0.5 nM). In a previous study, a K_d of approximately 450 pM for FDC-ER cells was reported (2). Thus, observed limited differences in affinities for wild-type versus mutant receptors indicate a possible contribution of WSAWSE and cytosolic domains to high-affinity binding.

TABLE 1. Cell surface EPO receptor expression of wild-type (ER) and mutant EPO receptors in FDC-P1 transfectants

Cell type	No. of EPO receptors/cell[a]
FDC-P1	None
FDC-ER	422 ± 151
FDC-WG	783 ± 172
FDC-2WG	823 ± 65
FDC-2WF	836 ± 331
FDC-2ST	800 ± 38
FDC-EKe[b]	506 ± 78
FDC-BS	336 ± 199

[a] Cells were incubated with an estimated saturating concentration of radiolabeled EPO (6 nM). On the basis of the amount of specifically bound ^{125}I-EPO, the numbers of cell surface receptors per cell were determined. The results are the averages of two independent experiments, including the standard errors. Each experiment included duplicate samples.
[b] FDC-EK cells maintained in EPO.

TABLE 2. Ligand-binding affinities for wild-type (ER) versus mutant EPO receptors

Sample	K_d[a] (nM)
FDC-ER cells	0.44 ± 0.24
	0.53 ± 0.14
FDC-ER membranes	0.56 ± 0.38
FDC-BS membranes	1.30 ± 0.60
COS-BS cells[b]	1.43 ± 0.28
FDC-WG cells	1.06 ± 0.25
FDC-2WF cells	1.66 ± 0.56
FDC-2ST cells	1.93 ± 0.30
FDC-EKe cells	1.25 ± 0.10

[a] Computed K_d values including standard errors.
[b] To increase receptor site densities, BS receptors were expressed transiently in COS cells.

Posttranslational processing of each receptor form in FDC cell sublines was assayed on the basis of receptor sensitivity to endo H (Fig. 4). Previously, three forms of wild-type EPO receptor have been reported, including an endo H-resistant, highly processed 66-kDa form, a 64-kDa endo H-sensitive form, and a minor 62-kDa endo H-resistant form (52). Immunoblotting of membranes isolated from FDC-ER and J2E-1 cells (an erythroleukemia cell line which differentiates in response to EPO) showed that each cell expressed these three receptor forms (M_rs of 66,000, 64,000, and 61,000). Although intracellular expression levels varied among receptors (e.g., lower expression of WG and 2WF receptors), glycosylation patterns similar to that of the wild type were seen for all receptor mutants except those expressed in FDC-2WG cells. In these cells, three to four endo H-resistant polypeptides were observed, indicating differential glycosylation, due possibly to altered polypeptide folding within the endoplasmic reticulum and Golgi apparatus. Altered patterns of glycosylation were also seen for truncated receptors in FDC-BS cells (data not shown). Interestingly, in FDC-2WG cells several low-molecular-mass receptor forms were observed, including one of 55 kDa. This 55-kDa recep-

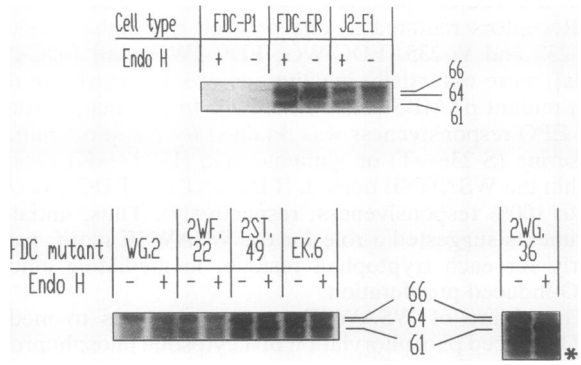

FIG. 4. Biosynthetic processing of mutated EPO receptors in FDC-P1 cells. Membranes isolated from FDC-P1 (negative control) and J2-E1 (positive control) cells and the indicated transfected FDC-P1 cells, including FDC-ER cells (positive control), were treated with (+) or without (−) endo H, subjected to SDS-PAGE (7.5%), and immunoblotted with anti-EPO receptor antibodies. Molecular size markers (in thousands) and the three glycosylated forms (66, 64, and 61 kDa) of full-length EPO receptors are indexed. The 55-kDa form of the 2WG receptor is denoted by an asterisk.

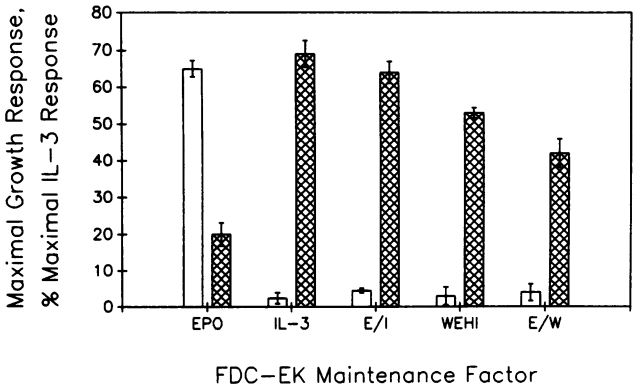

FIG. 5. Varied growth factor responsiveness levels of FDC-EK.6 cells maintained in EPO versus WEHI-3 versus IL-3. Induced [^{3}H]thymidine incorporation by 6 U of EPO per ml (open bars) or 200 pM GM-CSF (cross-hatched bars) was assayed and normalized by direct comparison with maximal growth rates induced by IL-3 (4 nM). The maximal dose responses are shown for FDC-EK.6 cells cultured in EPO (5 U/ml), 2% WEHI-3-conditioned medium (WEHI), synthetic IL-3 (2 nM), WEHI-3 plus EPO (E/W), and IL-3 plus EPO (E/I). The values are the means for triplicate samples ± standard deviations.

tor corresponds in molecular mass to the predicted size of an aglycosylated EPO receptor (6), suggesting that the 61- to 62-kDa form previously reported as a nonglycosylated form (52) in fact contains complex-type, endo H-resistant oligosaccharides.

Down-modulation of the EK receptor in IL-3- versus EPO-containing medium. Over the time course of these experiments (approximately 3 months), the responsiveness of FDC-EK cells (receptor mutant E-237→K) to EPO decreased substantially. As described in Materials and Methods, established FDC-P1 transfectants, once selected, were maintained in the presence of WEHI-3-conditioned medium as a source of IL-3. Periodically, the responsiveness to EPO, IL-3, and GM-CSF was assayed for all sublines expressing wild-type or mutant EPO receptors and was found to remain unchanged, with the exception of FDC-EK cells. Shown in Fig. 5 are the maximal proliferative responses to EPO or GM-CSF for clonal FDC-EK cells (subclone FDC-EK.6) maintained in EPO, WEHI-3, synthetic IL-3, WEHI-3 plus EPO, or IL-3 plus EPO. For FDC-EK.6 cells maintained in EPO, the responsiveness to EPO was comparable to that observed in control FDC-ER cells while growth induced by GM-CSF was minimal. Notably, in FDC-EK.6 cells maintained in WEHI-3, IL-3, or EPO in combination with maximal doses of WEHI-3 or IL-3, levels of EPO-induced growth were significantly reduced while responsiveness to GM-CSF approached levels exhibited by parent FDC-P1 cells.

EK receptor expression in FDC-EK cells cultured in WEHI-3, IL-3, or EPO was then examined (Fig. 6). When FDC-EK cells were maintained in WEHI-3 or IL-3, as opposed to EPO, levels of receptor expression were detectably decreased. Also, most receptors were processed only to the less glycosylated, 64-kDa form, which reportedly is not expressed on the cell surface (52, 53). Thus, IL-3 apparently affects the biosynthetic processing of expressed EK receptors, but the mechanism(s) by which EPO receptor expression is down-regulated has not been determined. Notably, posttranslational regulation of wild-type EPO receptors in 32D cells by EPO, IL-3, G-CSF, and GM-CSF has recently been reported (32).

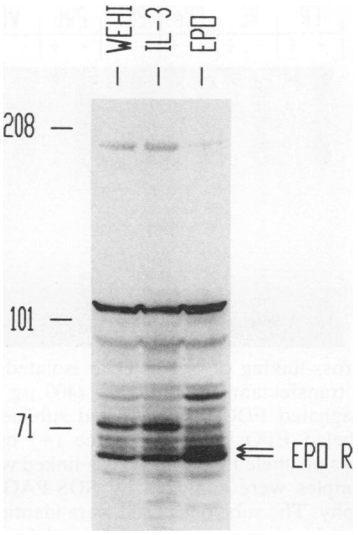

FIG. 6. Down-modulation of EK EPO receptor glycosylation and expression by IL-3. Membranes (400 μg per lane) isolated from FDC-EK.6 cells cultured in WEHI-3, synthetic IL-3 (2 nM), or EPO (5 U/ml) were subjected to SDS-PAGE (7.0% gel) and immunoblotted with anti-EPO receptor antibodies. The positions of molecular size markers (in thousands) and the two major forms of receptor (arrows [M_r, 66,000 and 64,000]) are indicated.

The WSAWSE region is essential for efficient endocytosis. A possible role for the WSAWSE and cytosolic domains of the EPO receptor in endocytosis was assessed by comparing the ability of wild-type versus mutant receptors to specifically bind and internalize ^{125}I-EPO (Fig. 7). Radiolabeled EPO was internalized efficiently by both FDC-ER and FDC-BS cells. In contrast, the ability of WSAWSE receptor mutants to internalize ^{125}I-EPO was either substantially reduced (receptor mutants WG, 2WG, 2ST, EKe, and EKw) or completely abrogated (receptor mutant 2WF). No specific

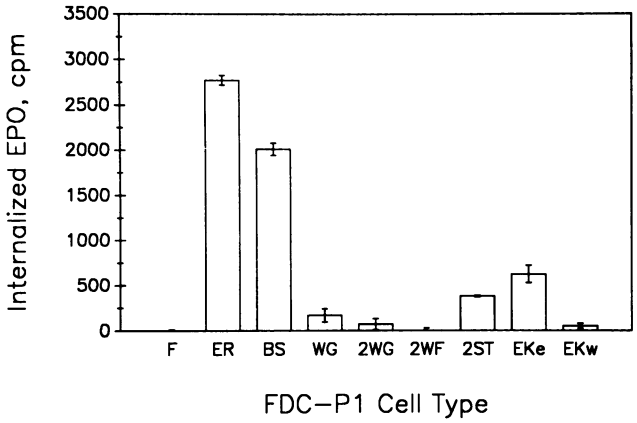

FIG. 7. Specific endocytosis of ^{125}I-EPO in cells expressing EPO receptor mutants. ^{125}I-EPO internalization among parent FDC-P1 cells (F) and transfected cells expressing wild-type (ER), mutated WSAWSE, or truncated (BS) EPO receptors was compared. Cell line designations and subclones are as designated in Fig. 1 and 2, except for FDC-EK cells cultured with EPO (EKe) or WEHI-3-conditioned medium (EKw). The values are the means for duplicate samples ± standard deviations. The mean background (nonspecific cell-associated counts) for all samples was 797 cpm (standard deviation = 87 cpm).

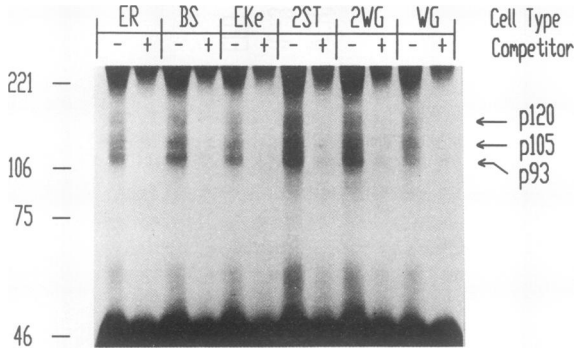

FIG. 8. Cross-linking of ^{125}I-EPO to isolated membranes from FDC-P1 cell transfectants. Membranes (400 μg per lane) isolated from the designated FDC-P1-transfected sublines were incubated with radiolabeled EPO in the presence (+) or absence (−) of 100-fold excess unlabeled EPO and cross-linked with disuccinimidyl suberate. Samples were analyzed by SDS-PAGE (7.5% gel) and autoradiography. The subclones used were identical to those in Fig. 2 and 3. Molecular size markers (in thousands) are indicated, as are the sizes of cross-linked proteins (determined by subtracting the M_r [35 kDa] of EPO).

internalization of EPO was detected in control FDC-P1 cells. These results indicate a role for the EPO receptor WSAWSE region in mediating efficient internalization of EPO. In contrast, the results for FDC-BS cells indicate that the cytosolic domain is dispensable for this function.

Cross-linking of EPO to accessory membrane proteins is mediated by intact and mutant EPO receptors. The possible involvement of the EPO receptor WSAWSE and cytosolic domains in mediating interactions with accessory proteins was assessed through the cross-linking of ^{125}I-EPO to membranes from FDC-P1 cells expressing wild-type and mutant receptors (Fig. 8). The results demonstrate that all EPO receptor forms assayed mediated the specific cross-linking of radiolabeled EPO to three membrane proteins with M_rs of 120,000, 105,000, and 93,000. Cross-linking of EPO to these proteins was inhibited by excess unlabeled hormone and was dependent on the expression of EPO receptors since no specifically cross-linked proteins were detected in control FDC-P1 cells (data not shown). Thus, while intact and mutated receptors differ in certain functional capacities, such as the ability to mediate growth signal transduction, all receptor forms studied nonetheless specifically associated with these three membrane proteins.

DISCUSSION

In the present studies, we have examined possible functional roles for the extracellular WSAWSE and cytosolic domains of the murine EPO receptor in growth signal transduction, binding and endocytosis, and receptor biosynthesis. Regarding the WSAWSE motif, our results suggest a critical role for each tryptophan residue in signal transduction, since receptors mutated at these regions fail to promote cell growth or the tyrosine phosphorylation of pp100. Conservation of serine and glutamate residues in this motif apparently is less critical, however, since S-236→T and E-237→K receptor mutants retain partial to full activity in each assay. By comparison, receptor mutants essentially lacking the predicted cytosolic domain were inactive. This is consistent with and extends previous studies which define a membrane-proximal cytosolic subdomain of approximately 80 to 90 residues to be necessary for efficient growth signal transduction (7, 33, 39).

Results indicating a strict requirement for tryptophan residues within the EPO receptor WSAWSE region are interesting to compare with those of recent studies of this motif in the related IL-2 receptor beta subunit (34). Specifically, the mutation of both tryptophans of the beta chain WSPWS domain to either serine or glycine abolished proliferative signaling, while mutation of both serines to glycine reduced responsiveness 1.5- to 3-fold. Each IL-2 receptor mutant was efficiently transported to the cell surface. However, neither tryptophan mutant bound IL-2 at detectable levels. Thus, for both IL-2 and EPO receptors, the conservation of tryptophans within this extracellular domain apparently is critical for signal transduction. Yet for the EPO receptor, our studies indicate that inactive WSAWSE tryptophan mutants retain the ability to specifically bind ligand. This was shown directly through whole-cell-binding analyses, as well as through the assessment of ^{125}I-EPO internalization. However, the ligand-binding affinities of all WSAWSE EPO receptor mutants were reduced (two to fivefold), regardless of the ability (2ST and EK receptors) or inability (WG and 2WF receptors) of these receptors to mediate EPO-induced proliferation. Thus, the EPO receptor WSAWSE domain is shown to be involved in ligand binding but apparently plays a more crucial role in receptor-mediated growth signaling. Interestingly, chimeric EPO/IL-3 receptors lacking the EPO receptor WSAWSE region yet possessing the IL-3 receptor WSEWS and cytosolic domains bind EPO and mediate EPO-induced growth (55). These results suggest that EPO-binding domains possibly are located amino terminal to the WSAWSE region in the EPO receptor and that the WSXWS and cytosolic domains of the IL-3 and EPO receptors are interchangeable for EPO receptor function. Notably, in contrast to our studies with myeloid FDC-P1 cells, it recently has been reported that WSAWSE EPO receptor mutants are inactive in mediating EPO-induced cell growth in lymphoid BA/F3 cells because of altered receptor processing and retention in the endoplasmic reticulum (49, 54).

The use of EPO-induced tyrosine phosphorylation of pp100 as an assay for the activity of WSAWSE receptor mutants merits brief discussion and is based on our recent identification of pp100 as a common cytosolic target for EPO-, IL-3, and GM-CSF-activated kinases (40). Importantly, we have shown in FDC-ER cells that (i) levels of induced pp100 phosphorylation correlate quantitatively with levels of EPO-induced cell proliferation and (ii) induced tyrosine phosphorylation depends on the expression of EPO receptor forms which are active in proliferative signaling (e.g., ER versus BS receptors). This applies to the presently studied WSAWSE mutants in that S-236→T and E-237→K receptors activate pp100 phosphorylation at detectable levels, while inactive tryptophan mutants do not. The precise role of pp100 in EPO-, IL-3, and GM-CSF-induced growth presently is undefined yet of considerable interest.

Since each mutated receptor form studied specifically bound EPO at the cell surface, the possibility that inactive forms are compromised in endocytosis was assessed. Endocytosis of receptor-EPO complexes has been observed to occur rapidly within 2 to 5 min and maximally at 45 to 60 min (7, 44), but no specific domains of the EPO receptor have been associated with this process. Our results indicate a role for the WSAWSE motif of the EPO receptor in endocytosis, since essentially all WSAWSE mutants studied internalized ligand at low levels compared with either wild-type or

truncated receptors. This includes partially active 2ST and EK mutants in addition to inactive WG, 2WG, and 2WF receptors, suggesting that EPO internalization may not be a limiting step in signal transduction. The observation that inactive BS EPO receptor forms (cytosolic deletion) internalize ligand efficiently likewise suggests (i) that ligand internalization and signal transduction may involve distinct and separate receptor domains, as observed for the IL-2 (18), insulin (46), and epidermal growth factor (38) receptors, and (ii) that EPO receptor endocytosis, in fact, may be mediated through transmembrane and/or extracellular domains. Previously, deletion of carboxy-terminal cytosolic domains of the IL-2 beta receptor and limited truncation of the murine EPO receptor cytosolic domain were shown to have no measurable effect on endocytosis (7, 18). This is in contrast to the well-characterized receptors for transferrin (16) and low-density lipoprotein (3), for example, in which specific cytoplasmic sequences and subdomains are necessary for receptor-ligand endocytosis.

While the EPO receptor cytosolic domain appears nonessential for ligand internalization, our results indicate that it is necessary for high-affinity ligand binding. Equilibrium binding analyses demonstrated a decrease of two- to threefold in EPO binding affinity for truncated BS versus wild-type ER receptors. This is in contrast to EPO receptor forms containing 72 membrane-proximal cytosolic amino acids or lacking cytosolic residues 9 through 30 (wild-type binding affinities, 400 to 800 pM) (33) and suggests that bulk deletion of the cytosolic domain may affect ligand binding, possibly by altering receptor folding or disrupting interactions with putative accessory proteins. For BS and 2WG EPO receptor mutants, altered glycosylation possibly reflects altered receptor folding, although this does not significantly affect receptor expression at the cell surface. By analogy, cytosolic deletions of the nerve growth factor receptor also lead to a loss of high-affinity ligand binding, possibly by disrupting interactions with trk (21). Notably, GM-CSF (20, 24), IL-2 (19), and IL-5 (45) receptors demonstrate high-affinity binding only when complexed with their respective second subunits. These examples differ from the prolactin and G-CSF receptor systems, in which deletion of cytosolic domains, respectively, enhances or has no effect on ligand binding properties (13, 42).

On the basis of studies that demonstrate the importance of conserved tryptophan residues in protein-protein associations (35) and on the basis of the homology between type III fibronectin domains of adhesion proteins and cytokine receptor extracellular domains containing the WSXWS motif, a role for the conserved WSXWS motif in mediating interactions between common or homologous receptor subunits has been suggested elsewhere (37). For the EPO receptor, no second subunit has conclusively been identified. However, the receptor has been found to associate with a phosphorylated 130-kDa protein (53), and cross-linking studies have shown that proteins of approximately 85 and 100 kDa form a multimeric complex with the receptor (8, 30, 31, 43). We presently show that the cloned EPO receptor mediates the specific cross-linking of ^{125}I-EPO to proteins with M_rs of 120,000, 105,000, and 93,000. This was also observed for all mutant receptors studied, including forms which were altered in their glycosylation (BS and 2WG) or were inactive in mediating growth signaling (BS, WG, and 2WG). Thus, neither the WSAWSE nor the cytosolic domains are essential for the association of EPO receptors with these proteins. Presently, it is not known whether these associated proteins are necessary for growth signal transduction (since they associate with inactive receptors) or whether mutant receptor-protein complexes are functionally inactive. Importantly, a recent report by Mayeux et al. demonstrates that none of the proteins cross-linked through disuccinimidyl suberate to ^{125}I-EPO in our studies corresponds to the EPO receptor itself (M_r, 66,000) (31). Control FDC-P1 cells do not mediate specific ^{125}I-EPO cross-linking, further demonstrating that the presently identified accessory proteins are EPO receptor associated. With regard to the 120-kDa cross-linked protein, it is intriguing that the shared subunit for the murine GM-CSF and IL-5 receptors (45) is of this size, especially since EPO receptors may compete with GM-CSF receptors in FDC-ER cells for a limiting component (e.g., receptor subunit) in their immediate pathways of induced growth (39).

In the present report, we also describe a unique EPO receptor mutant (E-236→K) which is inefficiently processed and is expressed at decreased levels when FDC-EK cells are cultured in IL-3 as opposed to EPO. Mechanistically, down-modulation of EK receptors by IL-3 is shown to be regulated, at least in part, at the posttranslational level by altered EPO receptor glycosylation. Similar regulation of EPO receptors recently has been observed in 32D cells, in which G-CSF, GM-CSF, and IL-3 inhibit cell surface expression of EPO receptors by altering their posttranslational processing and transport to the plasma membrane (32). By analogy, GM-CSF and IL-3 also have been shown to dominantly inhibit M-CSF receptor expression in FDC-P1/MAC cells at the posttranscriptional level (17). Biologically, the mechanisms which govern myeloid lineage restriction are thought to involve hierarchical down-modulation of growth factor receptors (48). Our results are consistent with this model yet also underline the importance of the growth factor environment in directly affecting the functional capacity of receptors already expressed within a cell.

In conclusion, the results shown here demonstrate a role for the membrane-proximal WSAWSE motif of the murine EPO receptor in ligand binding, intracellular processing, growth signal transduction, and endocytosis. We also show that removal of the EPO receptor cytosolic domain reduces ligand affinity and alters receptor glycosylation yet apparently does not affect endocytosis or receptor-mediated protein associations. Interestingly, equilibrium binding analyses indicate that the WSAWSE region is involved in, yet not essential for, EPO binding, and cross-linking experiments with disuccinimidyl suberate do not suggest a role for this domain in mediating receptor interactions with accessory proteins. In contrast, the WSXWS domain apparently is critical for ligand binding in the IL-2 beta and G-CSF receptors (13, 35). Thus, while this motif is essential for growth signal transduction in all systems studied to date, its specific function may vary among members of this cytokine receptor family. Studies of the WSXWS domain in additional members of the cytokine receptor family, such as the receptors for IL-3 and GM-CSF, should provide interesting and useful information regarding its significance in these structurally related yet functionally diverse receptors.

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