The Prolactin Receptor and Severely Truncated Erythropoietin Receptors Support Differentiation of Erythroid Progenitors*

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 Activation of the erythropoietin receptor is essential for the survival, proliferation, and differentiation of erythroid progenitors. To understand the role of erythropoietin receptor (EpoR) activation in erythroid differentiation, we infected primary erythroid progenitors with high-titer retrovirus encoding the non-erythropoietin prolactin receptor. The infected progenitors responded to prolactin in the absence of Epo by generating fully differentiated erythrocyte colonies. Therefore, differentiation of erythroid progenitors does not require an intracellular signal generated uniquely by the EpoR; the EpoR does not have an instructive role in erythroid differentiation. We also infected primary erythroid progenitors with retrovirus encoding chimeric receptors containing the extracellular domain of PrlR and the intracellular domain of either the wild-type or truncated EpoRs. A chimeric receptor containing only the membrane-proximal 136 amino acids of the EpoR cytoplasmic domain efficiently supported prolactin-dependent differentiation of erythroid progenitors. Substitution of the single cytoplasmic domain tyrosine in this receptor with phenylalanine (Y343F) eliminated its ability to support differentiation. The minimal EpoR cytoplasmic domain required for erythroid differentiation is therefore the same as that previously reported to be sufficient to support cell proliferation (D’Andrea, A. D., Yoshimura, A., Youssoufian, H., Zon, L. I., Koo, J. W., and Lodish, H. F. (1991) Mol. Cell. Biol. 11, 1980–1987; Miura, O., D’Andrea, A. D., Kabat, D., and Ihle, J. N. (1991) Mol. Cell. Biol. 11, 4895–4902; He, T.-C., Jiang, N., Zhuang, H., Quelle, D. E., and Wochowski, D. M. (1994) J. Biol. Chem. 269, 18291–18294).

The EpoR belongs to a large family of cytokine receptors, many of which are required for the proliferation and differentiation of hematopoietic as well as other cell types (4–7). Throughout life, eight different hematopoietic lineages arise from pluripotent stem cells in the bone marrow (8, 9). The exact role of growth factors in this process is not clear and has been described broadly by two alternative hypotheses. The stochastic hypothesis suggests that commitment of a progenitor to a particular lineage is a stochastic event, subsequent to which cell differentiation proceeds along a predetermined program; growth factors are merely required to ensure the survival and proliferation of committed progenitors (10–14). The contrasting inductive, or instructive, hypothesis attributes to growth factors a direct role in cell differentiation, predicting that growth factor receptors transduce signals that uniquely specify the differentiation outcome of a progenitor (15–17). A number of hybrid hypotheses have also been proposed, where, for example, committed progenitors arise stochastically, but their subsequent differentiation and acquisition of the mature phenotype are uniquely induced by lineage-specific growth factors (18).

Although Epo is essential for the production of red blood cells, it is not thought to play a role in the generation of committed erythroid progenitors from pluripotent progenitors: expression of recombinant EpoR does not bias the lineage commitment of pluripotent hematopoietic progenitors (19, 20), and normal numbers of committed erythroid BFU-e and CFU-e progenitors are found in the fetal livers of EpoR−/− mutant mice (21). However, there is a unique requirement for EpoR activation during the subsequent proliferation and terminal differentiation of committed erythroid progenitors: the EpoR−/− CFU-e and BFU-e progenitors fail to give rise to mature erythrocytes unless EpoR is expressed by retroviral infection (21); and in vitro, other growth factors only partially substitute for Epo (22–24). It is not known whether EpoR activation is essential at this stage of erythroid differentiation because of an EpoR-unique instructive signal or whether it is simply required for functions that are not unique to EpoR, such as its proliferative and anti-apoptotic effects.

Some evidence for the capability of EpoR to promote the erythroid phenotype comes from the ability of Epo to induce surface expression of glycophorin (25) and transcription of the β-globin gene (26, 27) in pre-B Ba/F3 cells expressing a transfected EpoR. However, the uncertain lineage commitment of many cell lines and their incomplete differentiation response makes them less suitable for the study of signaling in differentiation. EpoR-mediated signaling for proliferation can be studied in a number of interleukin-3-dependent cell lines, where heterologous expression of EpoR allows Epo to support cell proliferation (1, 2). Only the membrane proximal −120 amino acids is essential for this function (1–3). Similarly truncated mutants of other cytokine receptors are also able to support mitogenic signaling in such cells (17, 28–30). Since the greatest homology between cytokine receptors is found in the Box 1 and

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The abbreviations used are: Epo, erythropoietin; EpoR, erythropoietin receptor; Prl, prolactin; PrlR, prolactin receptor; BFU-e, burst-forming unit erythroid; CFU-e, colony-forming unit erythroid; IMDM, Iscove’s modified Dulbecco’s medium; IGF-I, insulin-like growth factor 1; FACS, fluorescence-activated cell sorting; MuLV, murine leukemia virus.
Box 2 domains of their membrane-proximal regions (30) (see Fig. 1), it might be expected that this region would generate signals for functions common to all these receptors such as cell survival and proliferation and that the divergent membrane-distal regions would endow the specificity of signaling presumed unique to each receptor.

We therefore examined whether the distal half of the EpoR cytoplasmic domain is essential for differentiation of primary fetal liver erythroid progenitors. We also examined whether the entirety of the cytosolic domain of the EpoR can be replaced with the corresponding segment of a different receptor; we chose the prolactin receptor, which plays no role in hematopoiesis, but belongs to the same subfamily of cytokine receptors as EpoR, and shares many of its signaling molecules.

**EXPERIMENTAL PROCEDURES**

_Chimeric Receptors—_CHI338 was generated by ligating a double-stranded oligomer containing a stop-codon in frame at position 339 of the EpoR between the BSP120I and HindIII sites of the murine EpoR cDNA (31). CHI374 was made by inserting a stop-codon in frame in the HindIII site of CHI. CHI374/Y343F was constructed by ligating a polymerase chain reaction product containing the point mutation into the BSP120I and HindIII sites of CHI374. CHI442 was made by subcloning the BSP120I-EcoRI fragment of pSFFV.tEpoR (32) into the BSP120I site of CHI. All receptors were expressed in the retroviral expression vector MSCV (33).

_Transducing Retroviruses—_The 293 cell line expressing the MuLV gag-pol proteins (34) was co-transfected with λenv, allowing the mouse ectropic MuLV envelope glycoprotein, a gift of Dr. David A. Sanders (Purdue University), and with SV2neo. Fifty G418-resistant clones were selected; of these, clone VE23 generated the highest titer of transducing retroviruses following transient transfection with a reporter MSCV DNA containing the lacZ gene. To generate transducing retroviruses, VE23 cells were transiently transfected using the calcium-phosphate method with MSCV retroviral constructs each encoding the desired receptor. Culture supernatants were collected at 48 h and either immediately frozen or used for infection.

_Fetal Liver Cell Infection and Culture—_Fetal livers from BALB/c mouse embryos were harvested at days E13 to 15 and dissociated by mechanical pipetting. The cells were subjected to a brief treatment with distilled water to lyse non-nucleated cells, strained through a 70-micron cell filter, and washed in Iscove’s modified Dulbecco’s medium (IMDM; purchased from Life Technologies Inc.) and 20% fetal calf serum. The cells were resuspended either in virus-containing culture supernatants containing 4 μg/ml polybrene or in control culture medium with 4 μg/ml polybrene. After rocking for 3–4 h at 37 °C, the cells were recovered by centrifugation. For CFU-e cultures, the cells were washed in IMDM and resuspended in serum-free methylcellulose medium (1% methylcellulose in IMDM, 1% bovine serum albumin, 100 ng/ml rIF-1, 7.7 μg/ml cholesterol, 5.6 μg/ml oleic acid, 5 μg/ml 1,2-phosphatidylcholine, 0.3 mg/ml transferrin, 4 μl/100 ml monothioglycerol) and ovine prolactin (the National Hormone and Pituitary Program of the NIDDK, National Institutes of Health, Bethesda, MD) as indicated. For BFU-e cultures, they were resuspended in 30% fetal calf methylcellulose medium (Stem Cell Technologies) with added spleen conditioned medium, 50 ng/ml rat stem cell factor, and 500 ng/ml ovine prolactin. Control (uninfected) cultures in each experiment had Epo added at 2 units/ml. Hemoglobinized BFU-e colonies were scored on day 3 after staining with diamino benzidine. Hemoglobinized BFU-e colonies were scored on day 7.

_FACS Scanning—_A small aliquot of cells from each infection was cultured in 20% fetal calf serum with 2 units/ml Epo and used at 36 h. The cells were washed three times in phosphate-buffered saline containing 0.5% BSA, incubated at 4 °C with 50 μg/ml monoclonal antibody M110 (gift of Dr. J. Dijane) in the presence of 200 μg/ml each of rabbit and goat IgG, followed by a phycoerythrin-conjugated goat anti-mouse F(ab’)2, Cells were scanned on a Becton-Dickinson cell scanner.

_Cytospins—_Five day 7 BFU-e bursts were individually aspirated from each methylcellulose culture. Following cytospin, cells were fixed in methanol, stained in acidine orange, and counterstained with Giemsa.

**RESULTS AND DISCUSSION**

_The Chimeric Receptor CHI, Containing the Full-length Cytoplasmic Domain of EpoR, Functions as Efficiently as the Endogenous EpoR in Supporting Erythroid Colony Development—_We used transient transfection of the packaging line VE23 to generate high-titer recombinant ectropic retrovirus and infected day 14 fetal liver cells in vitro with retroviruses encoding a series of chimeric receptors. These contained the extracellular domain of the rabbit prolactin receptor, which is not normally expressed by erythroid progenitors, and the transmembrane and cytoplasmic domains of EpoR (CHI (35)) or EpoRs truncated at amino acids 442 (CHI442), 374 (CHI374), or 338 (CHI338; Fig. 1). We then examined the ability of the infected fetal liver cells to generate erythroid colonies when cultured in semi-solid methylcellulose medium in the presence of prolactin (Fig. 2). In parallel, we measured the level of expression of the chimeric receptors by culturing a small aliquot of the infected fetal liver cells in liquid medium in the presence of Epo. After 36 h we incubated the cells with the monoclonal antibody M110, specific for the prolactin receptor (36), and quantified the fraction of M110-positive cells by FACS. The window we employed (M1, Fig. 2A) provides a lower estimate of the fraction of fetal liver cells infected by each of the chimeric receptors. Infection by CHI varied from 10 to 25% (Figs. 2, A, C, and D).

In each of five experiments, the ratio of prolactin-dependent erythroid BFU-e and CFU-e colonies formed by CHI-infected cultures to those formed by parallel uninfected and erythropoietin-treated cultures was the same or higher than the estimated rate of infection by CHI (Fig. 2). Specifically, Epo supported the generation of 700–1000 CFU-e colonies (per 2 × 10⁵ fetal liver cells; C); 10% of the fetal liver cells expressed CHI, and this population generated 100 CFU-e colonies promoted by prolactin. Thus the chimeric receptor CHI, containing the full-length cytoplasmic domain of EpoR, functions as efficiently as the endogenous EpoR in supporting erythroid colony development.

_CHI374 Contains a Minimal EpoR Domain Required to Efficiently Support Erythroid Differentiation; Tyr-343 Is Essential for This Function—_CHI442 and CHI374 were each able to support day 3 prolactin-dependent erythroid colonies in serum-poor medium (CFU-e-derived colonies) and day 7 erythroid bursts (BFU-e-derived bursts) to the same extent as CHI (Fig. 2, B and C). CHI338 was not able to support erythroid colony formation, although it was well expressed at the infected fetal liver cells (Fig. 2, B and D). No significant quantitative differences were seen between CHI, CHI442, or CHI374 in their response to prolactin (Fig. 2, B and C). The reduction in numbers of colonies and bursts at high concentrations of prolactin (Fig. 2, C and D) is expected, assuming the PrlR, a member of the same family of cytokine receptors as the Epo and growth hormone receptors, follows the same sequential dimerization mechanism (37). Importantly, the colony and burst size, mor-
Phenotype, and degree of hemoglobinization promoted by CHI,
CHI442, and CHI374 were similar to those promoted by Epo
acting through the endogenous EpoR (Fig. 3). Therefore,
CHI374 contains a minimal EpoR domain required for eryth-
roid differentiation. Tyrosine 343 within this domain is essen-
tial for erythroid differentiation, since its replacement in
CHI374 with phenylalanine completely abolished its ability to
support CFU-e differentiation (CHI374/Y343F, Fig. 2D). This

FIG. 2. Truncated chimeric receptors and the PrlR support
erthropoiesis. A and B, a representative experiment in which fetal
liver cells were infected with chimeric receptors and cultured for BFU-e
burst formation. A, FACS scan of fetal liver cells, cultured in 2 units/ml
Epo for 36 h postinfection and stained with the anti-PrlR monoclonal
antibody M110. The percent of cells with fluorescein isothiocyanate
(FITC) fluorescence within the range M1 is a lower estimate of the
extent of infection. B, corresponding BFU-e-derived bursts cultured in
methylcellulose in the presence of 500 ng/ml prolactin and no Epo.
Burst numbers represent mean ± S.E. of triplicate measurements,
scored on day 7 of culture. In parallel, and in the same experiment,
uninfected control methylcellulose cultures incubated in serum-free meth-
ylcellulose medium with 2 units/ml Epo generated an average of 90 bursts per 2 × 10^5 cells.

FIG. 3. Truncated chimeric receptors and the PrlR give rise to
erthroid colonies with normal morphology. A, CFU-e and BFU-e
colonies in methylcellulose. Cells were either not infected and cultured
in Epo or infected with the indicated receptor and cultured in prolactin.
Scale bar = 100 μm. Panels a–d, day 3 CFU-e colonies in serum-poor
medium. Colonies were stained with diaminobenzidine. Panels e–h, day
7 BFU-e bursts. B, cytopsin preparations of individually aspirated
BFU-e bursts showing normoblasts at different stages of differentia-
tion. Cells were incubated with diaminobenzidine, which stains cyto-
plasmic hemoglobin brown, and counterstained with Giemsa. Scale
bar = 10 μm. Panel a, uninfected cells cultured in Epo. Panels b and c,
PrlR-infected cells cultured in prolactin. r = reticulocyte; O = ortho-
chromatophilic normoblast; p = polychromatophilic normoblast; Oe =
orthochromatophilic normoblast in the process of exonucleation.
same tyrosine is essential for mitogenesis and is one of the four in the EpoR able to mediate STAT5 activation (35–41). The minimal EpoR cytoplasmic domain required to support erythroid differentiation is therefore the same as that previously found to be sufficient to support cell proliferation (1–3).

Wild-type PrlR Can Fully Support Differentiation of Erythroid Progenitors — The finding that CHI374 can fully support cell proliferation (35, 42). Figs. 2, further, we infected progenitors with retrovirus encoding the differentiation signal emanating from the distal part of EpoR; erythroid differentiation suggests that there is no essential erythroid progenitors. SH-PTP1, Grb2, and Ras, are also activated by other cytokine receptors (6, 7). Here we show that the unique outcome of EpoR signaling is not due to any unique signals it may generate, but instead is encoded in the cellular environment of erythroid progenitors. The specificity of EpoR function in erythroid differentiation is therefore a result of its unique expression by erythroid progenitors.

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