

Induction of erythroid proliferation and differentiation by a trophoblast-specific cytokine involves activation of the JAK/STAT pathway

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ABSTRACT

Pregnancy is characterized by increased erythropoiesis within maternal and fetal compartments. The placenta has been shown to produce factors that stimulate erythropoiesis but convincing evidence for placental production of erythropoietin (EPO) is still lacking. Prolactin-like protein E (PLP-E) was recently found to stimulate expression of the adult beta major globin gene in mouse erythroleukemia cells. Here we demonstrate that PLP-E transiently expressed in COS-7 cells stimulates proliferation and erythroid differentiation of murine and human erythroid progenitor cell lines. Electrophoretic mobility shift assays were used to show the activation of STAT5 by PLP-E in

the human erythroid cell line TF1. Furthermore, we compared the effects of PLP-E on murine myeloid FDCP1 cells which do not express EPO receptors (EPORs) with effects on cells genetically engineered to express functional EPORs. We provide evidence that PLP-E-dependent proliferation and STAT5 activation is independent of the expression of the EPOR. Taken together, these data suggest that PLP-E acts on specific receptors of erythroid-committed murine and human cells by the activation of intracellular signaling pathways promoting cell growth and differentiation.

Journal of Molecular Endocrinology (2000) **25**, 253–262

INTRODUCTION

Viviparity in mammals requires the dedication of maternal resources that go far beyond the limited provision with nutrients found in oviparous species. The formation of a separate ectodermal compartment, the trophoblast, and its subsequent assembly into a functional placenta represent pivotal developmental events. By establishing intimate contact with the maternal circulation, hemochorial placentation provides optimal gas exchange, supply of nutrients and waste disposal, but poses several challenges to the maternal organism and the developing fetus. Thus, the maternal response to pregnancy entails a variety of immunological, endocrine, metabolic and cardiorespiratory adaptations.

The placenta produces a wide array of hormones, cytokines and growth factors that are predicted to

manipulate maternal physiology for fetal benefit. In rodents, ruminants and primates, proteins with a close structural relationship to the pituitary hormones prolactin (PRL) and growth hormone (GH) are prominently expressed within the uteroplacental compartment (Soares *et al.* 1998a,b, Linzer & Fisher 1999). Although all members of the family bear structural similarity to pituitary PRL, only the classical members, placental lactogens (PLs), represent PRL receptor (PRL-R) agonists. All other proteins of the PRL gene family do not bind to the PRL-R and constitute the group of nonclassical members. The mouse placental PRL gene family was recently expanded by several nonclassical members: decidual PRL-related protein, PRL-like protein-A (PLP-A), PLP-B, PLP-C α , PLP-E, PLP-F and PLP-G (Lin *et al.* 1997a,b, Orwig *et al.* 1997, Dai *et al.* 1998, Müller *et al.* 1998a,b). This

expansion was accompanied by an increased elaboration of functional insights. In addition to the well-characterized actions of PLs, nonclassical members of the PRL family that cannot bind to the PRL-R have been shown to bind and affect specific targets such as endothelial cells (Jackson *et al.* 1994), uterine natural killer cells (Müller *et al.* 1999) and megakaryocytes (Linzer & Fisher 1999). Moreover, recent evidence suggests that PLP-E can stimulate erythropoiesis (Lin & Linzer 1998) and induces megakaryocyte differentiation (Lin & Linzer 1999).

The purpose of our investigation was to study the effects of PLP-E on proliferation and differentiation of murine and human erythroid progenitor cell lines and to examine the involvement of the erythropoietin (EPO) receptor (EPOR) and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway in the stimulation of erythropoiesis by PLP-E.

MATERIALS AND METHODS

Expression of PLPs in COS-7 cells

COS-7 cells were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM; Gibco, Eggenstein, Germany) containing 20 mM Hepes and supplemented with 10% fetal bovine serum (FBS). In this study, we used a cDNA for PLP-E which was originally independently characterized by two groups and published as PLP-E and PLP-G respectively (Lin *et al.* 1997b, Müller *et al.* 1998b). The cDNA clone for PLP-E (clone No. 557289, Genbank Accession AA111341, NID g1663220) was originally identified from the expression library 'Life Tech mouse embryo 10.5 dpc' and was obtained from IMAGE Consortium and Research Genetics, Huntsville, AL, USA (Lennon *et al.* 1996). The cDNA for PLP-A (Genbank Accession AF015562, NID g2353721), which was included for control purposes, has been described previously (Müller *et al.* 1998a). The cDNAs were cloned in the pCMV-SPORT2 eukaryotic expression vector (Life Technologies, Gaithersburg, MD, USA). Before introduction of the DNA into COS-7 cells, the sequences were completely verified by sequencing both strands.

Anion-exchange resin (Qiagen GmbH, Hilden, Germany)-purified nonlinearized plasmid DNA (20 µg) was electroporated into COS-7 cells using a Bio-Rad (Richmond, CA, USA) Gene Pulser (960 µF, 250 V). Transfections with the empty expression vector were included as controls. After 24 h of growth as adherent monolayers the medium was replaced by FBS-free culture medium and the

cells incubated for another 24 h. The conditioned culture medium was harvested, clarified by centrifugation (12 000 g), sterile-filtered (0.22 µm), and stored at -20 °C until used. Supernatants from three separate transfections were tested with similar results.

Generation of anti-peptide antibodies for mouse PLP-E

In order to evaluate the effects of PLP-E, protein expression in the transfected cells had to be confirmed. Therefore, we generated a specific immunological probe to mouse PLP-E. The 16 mer GATENVADYTLWSGLE (amino acids 195–210) was selected based on its relative hydrophilicity/hydrophobicity, the presence of β-turns and sequence comparison with other members of the PRL-GH family as previously described (Deb *et al.* 1989). Following synthesis of the oligopeptide and its coupling to the carrier keyhole limpet hemocyanin (KLH) by using 1-ethyl-3-(3',3'-dimethylaminopropyl)-carbodiimide hydrochloride, rabbits were immunized with the oligopeptide-KLH preparations (PepScan Immunoanalytik GmbH, Berlin, Germany). Blood was collected prior to immunization, as well as 6 and 12 weeks after immunization. Reactivity of the pre-immune sera and the anti-peptide antisera were characterized with a peptide ELISA as previously described (Deb *et al.* 1989).

Characterization of PLP-E protein expression by Western blotting

Supernatants of transfected COS-7 cells were diluted with one volume of SDS sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 1.5% SDS, 4.2% β-mercaptoethanol, 0.01% bromophenol blue) and boiled for 3 min. Proteins (50 µl of crude supernatant) were separated through 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-ECL; Amersham International, Amersham, Bucks, UK) as described previously (Bittorf *et al.* 1994). Membranes were blocked with 1% BSA and incubated with anti-PLP-E or anti-PLP-A antiserum (1:500). The PLP-A antiserum was described previously (Müller *et al.* 1998a). After a final incubation with a horseradish peroxidase-labeled anti-rabbit Ig antibody, blots were developed using the ECL system (Amersham).

Evaluation of PRL-like biological activity

Lactogenic biological activities were assessed using a rat Nb2 lymphoma cell proliferation assay

(Tanaka *et al.* 1980, Müller *et al.* 1998c). Nb2 lymphoma cells were routinely grown in Fischer's medium supplemented with 50 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% horse serum (HS) and 10% FBS (maintenance medium (MM)) in an atmosphere of 5% CO₂/95% air at 37 °C. Twenty-four hours before initiation of the assay, cells were harvested, washed with Fischer's medium containing only 10% HS (stationary medium (SM)), and diluted to a concentration of 100 000 cells/ml. At the initiation of the assay, cells were washed and transferred into 16 mm wells (100 000 cells/ml per well) of a 24-well culture plate. Ovine PRL (oPRL) or serum-free conditioned media from COS-7 cells transiently transfected with PLP-E-cDNA or empty vector were added at various concentrations and the cells incubated for an additional 72 h. Triplicate samples were collected and counted in a hematology analyzer (Cell-Dyn 1600; Abbott, Wiesbaden, Germany).

Hematopoietic cell lines

The murine erythroid progenitor cell line J2E (Busfield & Klinken 1992) was grown in suspension culture in DMEM (Gibco) supplemented with 10% FBS. Cells were resuspended daily at a density of 1.5×10^5 cells/ml. The human erythroleukemia cell line TF1 (Kitamura *et al.* 1989) was cultivated in RPMI 1640 (Gibco), supplemented with 10% FBS and 7.5 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Boehringer, Mannheim, Germany). The murine myeloid FDCP1 cells grow in the presence of interleukin-3 (IL-3) and are not responsive to EPO. The cells were propagated in Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% FBS. IL-3 was provided as conditioned supernatant (5% v/v) of WEHI 3B cells (Ymer *et al.* 1985). Parental FDCP1 cells were transfected by electroporation with the EPOR cDNA (D'Andrea *et al.* 1989) cloned into the expression vector pcDNA3 (Invitrogen Corp., Carlsbad, CA, USA). Stably transfected cells were selected in G418 (1 mg/ml) and by their ability to grow in response to EPO.

Recombinant EPO (Cilag GmbH, Sulzbach, Germany) and GM-CSF were used at concentrations of 5 U/ml and 10 U/ml respectively. These concentrations were found to result in a maximum stimulation of receptor activities. Unless otherwise stated, conditioned media from COS-7 cells were used at a final concentration of 20%. All experiments included controls using medium conditioned by COS-7 cells transfected with the empty expression vector.

Cell proliferation assay and benzidine staining

Cell proliferation was analyzed by [³H]thymidine incorporation. Cells (10^4 - 5×10^4) were pulsed with 0.6 µCi [³H]thymidine (83 Ci/mmol) for 4 h as described previously (Jaster *et al.* 1996). DNA was harvested onto glass fiber filters and assayed by liquid scintillation counting. Cell viability was assessed by trypan blue exclusion. Benzidine staining was used to determine the number of hemoglobin-synthesizing cells (Jaster *et al.* 1996).

Preparation of nuclear extracts and electromobility shift assay (EMSA)

Nuclear extracts were prepared essentially as described previously (Tilbrook *et al.* 1996). For EMSAs, nuclear proteins corresponding to 10^5 cells were incubated with 16 fmol double-stranded oligonucleotide derived from the bovine β-casein promoter (5'-AGATTTCTAGGAATTCAAATC-3'). The oligonucleotides were end-labeled with [^γ-³²P]ATP (Amersham) by polynucleotide kinase. The shift assays were performed in a total volume of 20 µl in the following buffer: 10 mM Tris-HCl (pH 7.5), 50 mM potassium chloride, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml BSA, 5% glycerol, 0.1% NP40, 1 mM Pefabloc (Boehringer). The reactions, also containing poly (dI-dC) 1 mg/ml (Boehringer), were performed at room temperature for 30 min and initiated by the addition of nuclear extract. Supershift analysis was done by including 0.5 µg of an antibody directed against the carboxy-terminal part of STAT5B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C for an additional 20 min. Complexes were analyzed by electrophoretic separation on a 6% polyacrylamide gel in 0.25 × TBE buffer.

Statistical analysis

Raw data were analyzed by one-way ANOVA. The source of variation from significant F ratios was determined with Student's two-tailed *t*-test. The Wilcoxon signed ranks test was used to examine percentage data. For all tests, the statistical software package SPSS 9.0 (SPSS Inc., Chicago, IL, USA) was applied. Differences were considered significant if $P < 0.05$.

RESULTS

Expression of recombinant PLP-E

Western blot analysis revealed that the transfection of COS-7 cells with the mouse PLP-E cDNA

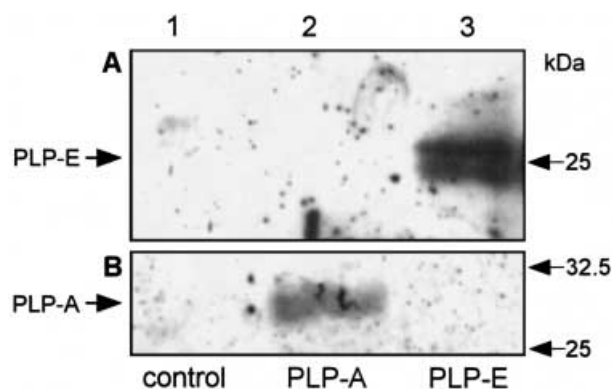


FIGURE 1. Demonstration of PLP-E protein expression by Western blotting. Supernatants of COS-7 cells transfected with mouse PLP-E cDNA, PLP-A cDNA and empty vector (control) were separated on a 10% SDS-polyacrylamide gel. Following transfer to a nitrocellulose membrane and blocking with 1% BSA, the membranes were incubated with rabbit antisera (1:500) to PLP-E (A) and PLP-A (B). After a final incubation with a horseradish peroxidase-labeled anti-rabbit Ig antibody, blots were developed using the ECL system.

resulted in the expression of the respective protein. The PLP-E protein was identified using an anti-peptide antibody raised against amino acids 195–210

of the mouse PLP-E sequence (Fig. 1A). The antibody detects a doublet of proteins, which is probably caused by the expression of differentially glycosylated isoforms of PLP-E. To verify the specificity we analyzed the supernatants with an antibody directed against PLP-A (Fig. 1B). This antibody detects an immunoreactive protein species in PLP-A but not in PLP-E or control supernatants.

PLP-E does not signal via the PRL-R

To exclude the possibility that PLP-E activates the PRL-R, we tested the effect of the recombinant protein on rat Nb2 lymphoma cells. PLP-E was not capable of stimulating the proliferation of rat Nb2 lymphoma cells (Fig. 2). Rat Nb2 lymphoma cell proliferation is dependent on activation of the PRL-R signaling pathway, which can be achieved by PRL or other ligands capable of interacting with the PRL-R, such as PLs (Tanaka *et al.* 1980, Müller *et al.* 1998c).

Effects of PLP-E on the proliferative response of hematopoietic cell lines

The growth-stimulating activity of PLP-E was analyzed by [³H]thymidine incorporation assays.

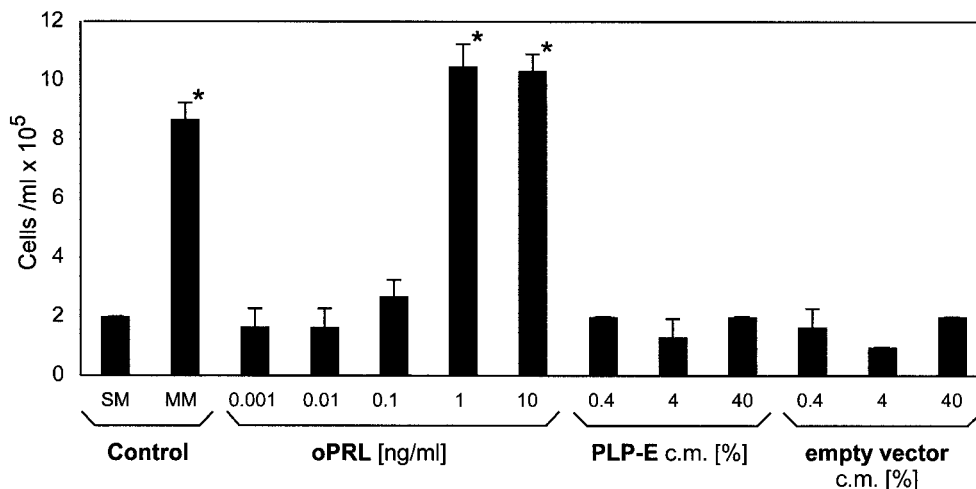


FIGURE 2. Effects of PLP-E on rat Nb2 lymphoma cell proliferation. PRL-like biological activities were assessed by use of the rat Nb2 lymphoma cell proliferation assay. Nb2 lymphoma cells were grown in MM. Twenty-four hours before initiation of the assay, cells were harvested, washed with SM, and diluted to a concentration of 100 000 cells/ml. At the initiation of the assay, cells were washed and transferred into 16 mm wells (100 000 cells/ml per well) of a 24-well culture plate. oPRL, conditioned medium (c.m.) of COS-7 cells transfected with PLP-E, or empty vector was added at various concentrations and the cells incubated for an additional 72 h and then counted. Treatments were performed in triplicate. Results are presented as means \pm s.d.. The oPRL-treated cultures show significantly stimulated proliferation of the Nb2 lymphoma cells compared with control cells in SM (* $P < 0.05$). Note that PLP-E did not stimulate Nb2 proliferation.

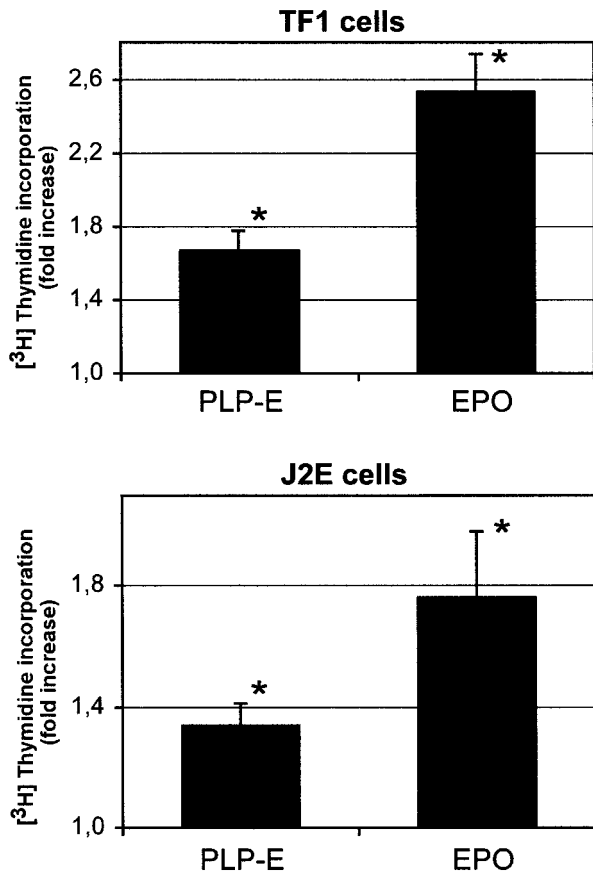


FIGURE 3. PLP-E-dependent proliferation in J2E and TF1 cells. Cells were grown in the presence of PLP-E-conditioned media or EPO (5 U/ml) and pulsed with [³H]thymidine as described. Results indicate the ratio of values derived from stimulated cells vs control values (cells incubated with supernatant of COS-7 cells transfected with an empty vector) and represent the mean of five independent experiments. Results are presented as means \pm S.E.M. Note that EPO and PLP-E significantly stimulated the proliferation of murine and human erythroid progenitor cells (* P <0.05).

Both human (TF1) and mouse (J2E) erythroleukemic cell lines were examined. TF1 cells are known to be responsive to a panel of cytokines, including EPO, IL-3 and GM-CSF, whereas the J2E cells can grow independently of the addition of exogenous cytokines but show an increased proliferation rate after addition of EPO (Busfield & Klinken 1992). Furthermore, J2E cells as well as TF1 cells differentiate along the erythroid pathway upon treatment with EPO. The effects of PLP-E-containing conditioned COS-7 media were normalized using the supernatant of COS-7 cells transfected with the empty vector (control; Fig. 3).

EPO-induced responses are shown as a positive control.

In J2E cells, recombinant EPO and PLP-E (but not the related PLP-A; data not shown) showed significant growth-promoting activities (Fig. 3). In TF1 cells, we found a similar response to both factors (Fig. 3). The results shown were calculated using data from five assays and were reproducible with supernatants produced by independent transfections.

PLP-E induces erythroid differentiation of J2E and TF1 erythroleukemic cell lines

To further investigate the biological effects of PLP-E on hematopoietic cells we analyzed its ability to induce erythroid maturation. Both J2E and TF1 cells synthesize hemoglobin and undergo morphological alterations in response to EPO (Gobert *et al.* 1996, Lin *et al.* 1996). Hemoglobin-containing cells were detected by benzidine staining. PLP-E induced a significant increase of hemoglobin synthesis in both cell lines, similar to the EPO-induced response (Fig. 4).

PLP-E induces the DNA-binding activity of STAT5 in TF1 cells

Many cytokines are known to induce the JAK/STAT pathway by the activation of specific sets of JAK tyrosine kinases and transcription factors of the STAT family. In view of the biological activities of PLP-E seen above we were interested in its ability to activate this pathway. Nuclear extracts from PLP-E as well as cytokine-stimulated TF1 cells were prepared and subjected to EMSA using a STAT binding site derived from the β -casein promoter. As shown in Fig. 5, EPO, GM-CSF and PLP-E induced a DNA-protein complex. In an attempt to identify the proteins of the complex, we employed supershift analysis using an antibody specific for the carboxy-terminal domain of STAT5b. All induced complexes undergo a complete mobility shift indicating the presence of STAT5 whereas antibodies to other STAT species (STAT1, 3) display no interaction (data not shown). The induction of STAT5 DNA-binding activity is not restricted to TF1 cells since the analysis of J2E cells revealed qualitatively identical results (data not shown).

Induction of proliferation and STAT5 activation by PLP-E is independent of the expression of the EPOR

The effects of PLP-E on the proliferation and differentiation of erythroleukemic cell lines documented

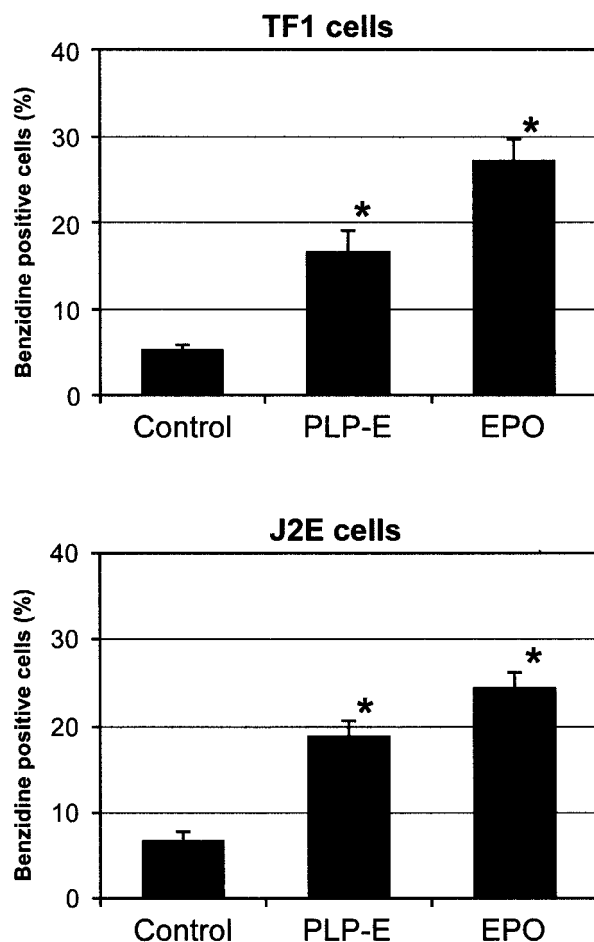


FIGURE 4. PLP-E-dependent erythroid differentiation in J2E and TF1 cells. Cell cultures were established at 10^5 cells/ml and exposed to EPO (5 U/ml) or PLP-E-conditioned media as described. Benzidine-positive cells were determined 48 h later. These data are calculated from five independent experiments and are presented as means \pm S.E.M. Note that EPO and PLP-E significantly stimulated the erythroid differentiation of murine and human erythroid progenitor cells (* $P < 0.05$).

above are very similar to EPO-induced biological activities. Therefore, it is tempting to speculate that PLP-E might activate the EPOR itself to induce erythroid-specific cellular reactions. To investigate this possibility we used IL-3-dependent FDCP1 cells, which do not express endogenous EPORs (FDCP1 WT), and compared them with cells stably transfected with the EPOR cDNA (FDCP1 EPOR). Figure 6 shows the results of proliferation assays using both cell types. As expected, EPOR-expressing cells respond to EPO by enhanced proliferation and display the same IL-3-dependent proliferation as untransfected

FDCP1 cells. PLP-E significantly enhances the [3 H]thymidine incorporation in FDCP1 WT cells, indicating that this factor exerts its effects independently of the EPOR expression. Interestingly, the mitogenic effect of PLP-E is reduced in EPOR-transfected cells.

Additional experiments were done to extend these data to the intracellular signaling capabilities of both cell types (Fig. 7). EMSA revealed that PLP-E is a potent activator of STAT5 both in FDCP1 WT and in FDCP1 EPOR cells. In contrast, EPO induces STAT5 activation in FDCP1 EPOR cells only, providing further evidence for the EPOR-independent action of PLP-E.

DISCUSSION

Hematopoiesis is controlled by a group of polypeptide ligands which signal through a structurally related set of transmembrane receptors to regulate the lineage-specific development of progenitor cells (Moritz *et al.* 1997). These cytokines induce specific signaling pathways to promote viability, proliferation and differentiation of hematopoietic cells. During embryonic development, erythropoiesis occurs in two distinct forms (McGann *et al.* 1997, Silver & Palis 1997). In the mouse, the first 'primitive' form is represented by nucleated erythroblasts that begin to differentiate within the blood vessels of the extra-embryonic yolk sac on the eighth gestational day. The second 'definitive' form of erythropoiesis is thought to originate from hematopoietic progenitors that emerge from endothelial cells in the yolk sac, the vitelline and umbilical arteries, and in the aorta/genital ridge/mesonephros region. Beginning on gestational day 10, anucleate erythrocytes differentiate within the fetal liver. Towards term, the bone marrow of the fetus becomes the dominant site of erythropoiesis and the production of red cells is approximately 3–5 times that of adult steady state levels (Moritz *et al.* 1997, Silver & Palis 1997). A significant increase in blood volume characterizes maternal adaptation to pregnancy. In the human, this is reflected by a 50% increase in plasma volume, an 18–25% increase in red cell mass and 4-fold increased EPO levels (Letsky 1995).

The glycoprotein EPO is synthesized in the fetal liver and the adult kidney and has been shown to be the primary growth factor involved in the regulation of red blood cell production (Moritz *et al.* 1997). Gene targeting studies revealed that both EPO and EPOR genes are essential for definitive erythropoiesis *in vivo* (Wu *et al.* 1995, Lin *et al.* 1996). It is well established that maternal EPO does not cross the placenta into the fetal compartment (Schneider

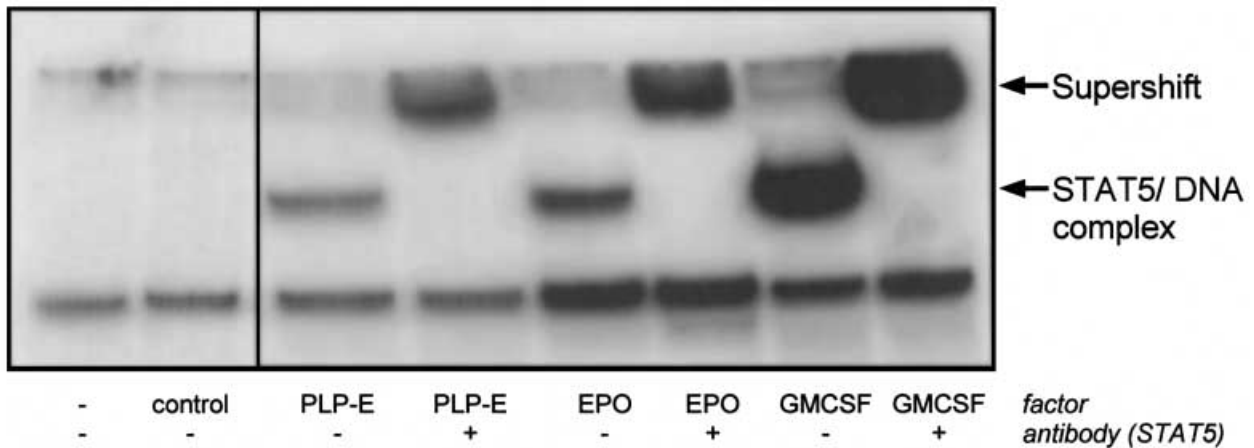


FIGURE 5. Activation of STAT5 DNA-binding activity in PLP-E- and cytokine-stimulated TF1 cells. Cells were deprived of GM-CSF for 24 h and stimulated for 15 min with EPO (5 U/ml), GM-CSF (10 U/ml), or serum-free conditioned medium of COS-7 cells transfected with PLP-E or empty vector. Nuclear extracts were prepared and subjected to EMSA as described. Note that PLP-E, EPO and GM-CSF induced the formation of a protein–DNA complex that could be completely supershifted by an antibody to STAT5.

& Malek 1995). However, expression of the EPOR in trophoblast cells from murine and human placenta has been shown at the mRNA and protein level (Sawyer *et al.* 1989, Fairchild & Conrad 1999). Although EPO immunoreactivity was demonstrated in human placenta, its mRNA expression could not be confirmed by Northern blotting (Conrad *et al.* 1996). It is therefore assumed that the positive immunostaining represents EPOR-bound EPO (Moritz *et al.* 1997).

Trophoblast cells produce factors that stimulate erythropoiesis (Brewer *et al.* 1992). Recent evidence suggests that members of the PRL gene family are involved in this activity (Lin & Linzer 1998). The placental PRL gene family represents a group of structurally related proteins specifically expressed during pregnancy by trophoblast cells of the placenta and/or adjacent decidua (Soares *et al.* 1998b). Several members of this family (PRL and PLs) signal via the PRL-R. The PRL-R as well as the receptors for GH and thrombopoietin (TPO) belong to the class I cytokine receptor family and are able to substitute for the EPOR in erythroid development (Golde *et al.* 1977, Kieran *et al.* 1996, Socolovsky *et al.* 1998). It seems therefore possible that members of the PRL family utilize signaling mechanisms that evolved from class I cytokine receptor prototypes. The presented data show that PLP-E of the mouse placenta induces proliferation and differentiation of erythroid precursors. These effects were demonstrated in murine and human cell lines. PLP-E is produced in the mouse placenta by trophoblast giant cells (Lin *et al.* 1997b, Müller

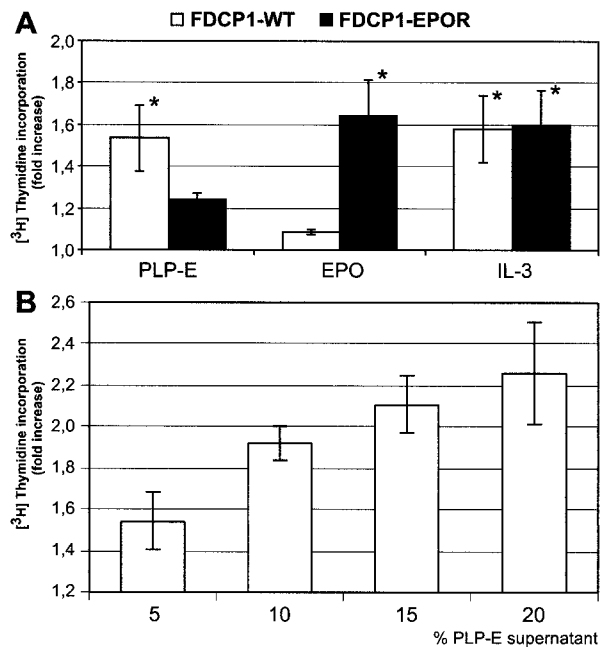


FIGURE 6. Comparative analysis of FDCP1 WT and FDCP1 EPOR cells. (A) [³H]thymidine incorporation after stimulation with PLP-E-conditioned media, EPO and IL-3. Results indicate ratios of values derived from stimulated cells vs control values and represent the means ± s.e.m. of five independent experiments. EPOR-expressing cells respond to EPO by enhanced proliferation and display the same IL-3-dependent proliferation as untransfected FDCP1 cells. Note that PLP-E significantly stimulated proliferation in wild-type FDCP1 cells that lack the EPOR (**P*<0.05). (B) FDCP1 WT cells were stimulated with different amounts of PLP-E supernatant and analyzed as above.

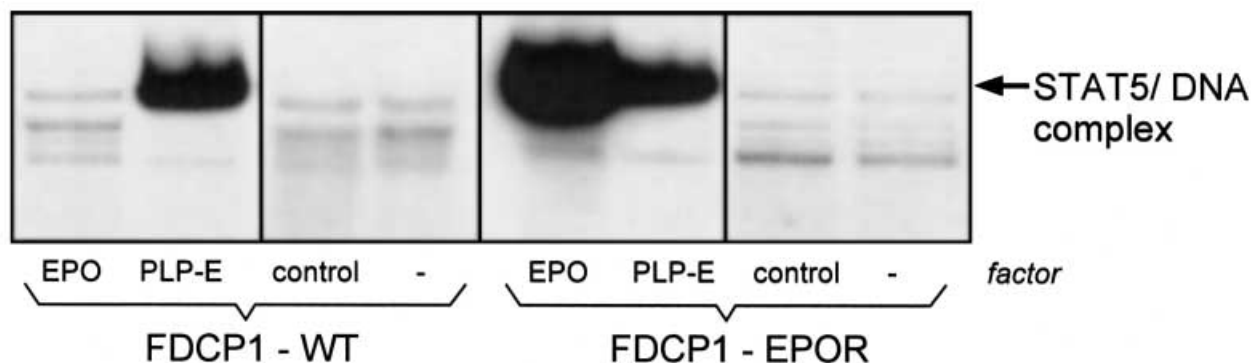


FIGURE 7. PLP-E- and EPO-induced activation of STAT5 DNA-binding activity in FDCP1 WT and FDCP1 EPOR cells. Cells were deprived of IL-3 for 24 h and stimulated for 15 min with EPO (5 U/ml) or serum-free conditioned medium of COS-7 cells transfected with PLP-E or empty vector. Nuclear extracts were prepared and subjected to EMSA as described. Note that PLP-E induced the formation of a STAT5/DNA complex in wild-type and EPOR-transfected FDCP1 cells whereas EPO activates STAT5 only in FDCP1 cells that express the EPOR.

et al. 1998b). Within the uteroplacental compartment, PLP-E is positioned to potentially affect maternal as well as fetal hematopoietic targets. This dual direction of transport has been demonstrated for the PRL family member proliferin (PLF), which is also secreted by trophoblast giant cells. PLF associates with the yolk sac and has been shown to be present in maternal serum and in fetal compartments (Lee *et al.* 1988, Jackson & Linzer 1997). Thus, PLP-E may very well affect yolk sac erythropoiesis. This speculation is indirectly supported by the recent observation showing that yolk sac erythropoiesis is not exclusively dependent on EPO (McGann *et al.* 1997).

To further elucidate the molecular basis of PLP-E action, we studied the induction of signaling events known to be involved in cytokine-mediated gene activation in hematopoietic cells. Our data show that PLP-E induces the DNA-binding activity of STAT5, a transcription factor which has been shown to be a part of the EPO-dependent signaling machinery (Gouilleux *et al.* 1995, Liu *et al.* 1995). The essential role of STAT5 (which was originally identified as a mediator of lactogenic signaling (Liu *et al.* 1995)) in fetal hematopoiesis has been established using STAT5a^{-/-}/5b^{-/-} mouse embryos (Socolovsky *et al.* 1999), which are severely anemic. In contrast, adult mice that are nullizygous for STAT5 show apparently normal hematopoiesis (Teglund *et al.* 1998). It has been reported that EPORs defective in STAT5 activation are deficient in supporting mitogenesis (Gobert *et al.* 1996) and that a constitutively active STAT5 mutant promotes cytokine-independent proliferation after transfection in IL-3-dependent cells (Onishi *et al.* 1998). The activation of STAT5, however, is not

unique to the EPOR (Teglund *et al.* 1998). Several hematopoietin receptors and even nonhematopoietic receptors activate STAT5. Recent studies using receptor hybrids composed of the extracellular EPOR domain and the cytoplasmic part of the GH receptor, the TPO receptor and the GM-CSF receptor show that these receptors activate appropriate signal transduction pathways to support erythroid development *in vitro* and *in vivo* (Goldsmith *et al.* 1998). Moreover, the receptors for PRL, GH and TPO, like the EPOR, belong to the class I cytokine receptor family, form homodimers upon activation, activate STAT5, and are able to substitute for EPOR in erythroid development (Golde *et al.* 1977, Kieran *et al.* 1996, Goldsmith *et al.* 1998, Socolovsky *et al.* 1998). Our experiments using EPOR expressing/nonexpressing cells provide evidence that the EPOR is not involved in the PLP-E-dependent STAT5 activation. The molecular background of the reduced mitogenic response in PLP-E-stimulated FDCP1 EPOR cells, which may occur at the level of intracellular signal transduction, remains to be established.

In conclusion, we hypothesize that PLP-E utilizes receptors that are functional in activating erythroid-specific pathways independently of the EPO/EPOR system and that these structures are potentially conserved.

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REVISED MANUSCRIPT RECEIVED 8 June 2000