Maturation of megakaryocytes and subsequent platelet release are normally regulated by a network of cytokines, including thrombopoietin and various interleukins. Because abnormal platelet production and activation have been implicated in gestational pathologies, additional pregnancy-specific cytokines may play important roles in the regulation of megakaryocytopenesis. Consistent with this hypothesis, we have found that the hormone prolactin-like protein E, a placental hormone that we have recently characterized, targets megakaryocytes through a specific cell surface receptor and induces megakaryocyte differentiation through a gp130-dependent signal transduction pathway.

Coordinated physiological changes during pregnancy are critical for mammalian reproduction. Many pregnancy-associated diseases such as pregnancy-induced hypertension, pre-eclampsia, and diabetes are the consequences of aberrant modulation of maternal physiology. Higher rates of platelet activation in plasma have been clinically linked to pre-eclampsia, and antiplatelet treatment has been widely used to treat patients with this disorder (1–3). The development of megakaryocytes (MK),1 release of platelets into the circulation, and activation of platelets are tightly regulated processes (4), and in rodents megakaryocytopenesis and platelet levels are elevated throughout gestation (5, 6). Although the platelet count has been found to decrease slightly in human pregnancy in some studies (7), the actual rates of platelet production apparently increase to compensate for the dramatic increase in blood volume during pregnancy (8).

An accelerated production of platelets in gestation presumably depends on higher levels of thrombopoietic factors present in the maternal plasma. Many cytokines, including thrombopoietin (TPO), interleukin-6 (IL-6), IL-11, leukemia inhibitory factor, and kit ligand, have been demonstrated to enhance MK maturation to different extents under normal physiological conditions (4, 9). It is unclear, however, what factors are responsible for the pregnancy-associated thrombopoietic activity. In mammalian pregnancy, the placenta functions as a transient endocrine organ that secretes a number of factors and modulates numerous physiological processes to accommodate the needs of the developing fetus.

Among the factors synthesized by the placenta are numerous proteins in the prolactin (PRL)/growth hormone family (10, 11). These proteins target distinct maternal tissues and exert various biological effects, such as the remodeling of the vascular network in the mouse by proliferin and proliferin-related protein (12), the regulation of steroid hormone production and metabolism by the placental lactogens (11), and the control of immune response by PRL-like protein A (13). As members of the cytokine superfamily, these placental hormones may act on a variety of hematopoietic cell types. We have therefore begun to search for the targets and physiological effects of these proteins with the expectation that some of these hormones will be found to be responsible for eliciting pregnancy-specific changes in the hematopoietic system.

Previously, we and others identified a novel placental glycoprotein hormone in the mouse PRL family, PRL-like protein E (PLP-E), which is expressed at high levels at midgestation in the mouse (14, 15). In this study, we show that PLP-E binds to mouse MK cells through a specific surface receptor and enhances MK differentiation through a gp130-dependent signaling pathway. Thus, PLP-E is a novel placenta-derived thrombopoietic activity that contributes to the pregnancy-specific changes in MK development and platelet production.

EXPERIMENTAL PROCEDURES

Preparation of Fusion Proteins—The PLP-E cDNA was linked in-frame to a secreted alkaline phosphatase (AP) gene in a mammalian expression vector described previously (16). Fusion protein was obtained by transient transfection of the DNA construct into Chinese hamster ovary cells and subsequent collection of culture medium over a 2-day period. Medium containing secreted AP-PLP-E was concentrated and used in binding assays. PLP-E without the AP fusion partner was also generated by similar approaches and used as a competitor in binding experiments. The PLP-E cDNA was also fused to a GST coding sequence to produce and purify GST-PLP-E fusion protein from bacteria.

Hormone Binding Assay—Tissue sections were prepared, and binding assays were carried out essentially as described (16). Briefly, sections were preincubated with appropriate competitor for 30 min at room temperature before incubating with AP-PLP-E for 45 min. Slides were washed briefly in Hanks’ balanced salt solution three times and fixed in a solution containing 20 mM HEPES (pH 7.4), 60% acetone, and 3% formaldehyde. After inactivating endogenous AP at 65 °C for 30 min, the enzymatic activity derived from the fusion protein was detected by a chromogenic reaction. In some experiments, adjacent sections were also stained for acetylcholinesterase (AchE) activity or with a monoclonal antibody that recognizes CD41 (17). Images were captured using a digital camera.

Colonies were counted using a digital camera.

Colony Formation Assay—Femurs from CD1 female mice were flushed with 5 ml of Iscove’s modification of Dulbecco’s medium containing 10% fetal bovine serum (Life Technologies, Inc.). The marrow cells were passed through gauge 19 and 25 needles sequentially and cultured at 37 °C for 45 min to remove attached stromal cells. The cells were washed with Iscove’s modification of Dulbecco’s medium containing 1% Nutridoma (Roche Molecular Biochemicals) and plated in semi-
solid medium for colony formation assays or liquid medium for flow cytometric analysis. Colony formation assays were performed using MegaCult medium (Stem Cell Technology, Vancouver, Canada) following instructions provided by the manufacturer. About 5 x 10^4 nucleated bone marrow cells were cultured for 5–6 days in each well on 2-well chamber slides. Typically, 10 ng/ml murine IL-3, 15 ng/ml murine IL-6, 50 ng/ml murine TPO, and 4 μg/ml GST or GST-PLP-E were included in the treatments. In some experiments, a mixture of monoclonal antibodies (RX187 and RX435) against gp130 (18), provided by Dr. Tetsuya Taga, was added to the cultures at a concentration of 5 μg/ml. Colonies were dried and stained for AchE activity before processing for microscopy and colony scoring. A MK colony was defined as at least three clustered positively stained cells.

**Flow Cytometric Analysis**—Bone marrow cells (5 x 10^6) were cultured in the presence of 3 ng/ml IL-3 with or without 2 μg/ml GST-PLP-E for 4–5 days. Cells were harvested and stained with fluorescein isothiocyanate-labeled anti-mouse CD41 (Pharmingen, San Diego, CA) for 60 min on ice. Cells were washed three times with cold phosphate-buffered saline and resuspended in a 0.1% citrate solution containing 50 μg/ml propidium iodide for 30 min before the addition of RNase to a final concentration of 20 μg/ml (19). Samples were stored at room temperature for 30 min in the dark before analysis on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). A mouse erythroleukemia cell line (GM979) was used as a ploidy control.

**Immunoblot Analysis**—A polyclonal antiserum against PLP-E was generated by immunization of rabbits with bacterially derived GST-PLP-E. Plasma samples from pregnant mice were collected on day 10 of gestation in Microtainers containing lithium heparin (Becton Dickinson). Samples were separated on 10% SDS polyarylamide gels and transferred to nitrocellulose membranes. After incubating in blocking buffer (20 mM Tris (pH 7.6), 150 mM NaCl, 0.5% Triton X-100, and 5% nonfat milk), the antiserum was added at 1:2000 dilution. After washing in blocking buffer, membranes were subsequently incubated with a secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG, and processed for film exposure.

**RESULTS**

**PLP-E Targets Mouse MK Cells**—To identify potential physiological targets for PLP-E action, a fusion protein containing AP and PLP-E was used as a probe to search for hormone binding sites in pregnant mouse tissues. AP-PLP-E binding was observed in most hematopoietic tissues, including fetal liver (data not shown), maternal bone marrow (Fig. 1A), and maternal spleen (Fig. 1B). Two distinct binding targets were identified in the spleen: large cells with multilobulated nuclei and abundant cytoplasm, characteristics of MK cells, and smaller binding targets that may correspond to cell fragments, in particular platelets. To confirm the identity of the large cells, adjacent spleen sections were prepared and stained for AchE, an enzymatic marker for the MK lineage. AchE activity colocalized to the cells that bound AP-PLP-E (Fig. 1C and D). Additionally, a monoclonal antibody (CD41) that reacts with mouse glycoprotein Ib, a MK-specific cell surface integrin, also recognized PLP-E target cells (data not shown). Interestingly, tissues from nonpregnant female and male mice displayed similar binding patterns for the fusion protein (data not shown). Therefore, although the synthesis of PLP-E is restricted to the mouse placenta in early gestation, the binding sites on MK cells appear not to be pregnancy-specific.

The addition of excess PLP-E completely eliminated binding by AP-PLP-E, whereas another PRL-related placental hormone, PLP-B, was unable to block AP-PLP-E binding to MK cells (Fig. 1E and F). Thus, binding of PLP-E to MK cells is saturable and specific and therefore unlikely to involve MK cell-specific cell surface receptors. Similar to PLP-E produced in mammalian cell cultures, a bacterially derived fusion protein, GST-PLP-E, at 10 μg/ml effectively competed for PLP-E binding sites (Fig. 1G and H), indicating that specific binding does not depend on PLP-E glycosylation.

**PLP-E Enhances MK Cell Differentiation**—Because GST-PLP-E is capable of receptor binding and is readily purified in large amounts, this fusion protein was used to examine PLP-E activity. In a semi-solid culture, GST-PLP-E induced primary mouse bone marrow MK differentiation as shown by increased cellular size and enhanced AchE staining (Fig. 2, A and B), an effect comparable with that induced by the positive controls IL-6 or TPO (Fig. 2, C and D). To assess the induction of MK differentiation quantitatively, semi-solid primary mouse bone marrow cell cultures were supplemented with GST-PLP-E and IL-3, a cytokine capable of inducing proliferation of multiple hematopoietic lineages including MK progenitors (4). GST-PLP-E induced a dose-dependent increase in colony-forming units-megakaryocyte (CFU-MK), with significant effects observable at 1 and 4 μg/ml (Fig. 3). Although the effective doses of GST-PLP-E are higher than the optimal concentrations for IL-6 or TPO in this assay, these levels of PLP-E are comparable with the maternal plasma concentration of PLP-E during pregnancy; based on semi-quantitative immunoblotting and comparison to purified GST-PLP-E standards, plasma PLP-E is present at a concentration in excess of 1 μg/ml on day 10 of gestation (Fig. 4).

In addition to quantifying the effect of PLP-E based on colony formation, two-color flow cytometric analysis indicated that the addition of GST-PLP-E along with IL-3 in liquid medium cultures significantly shifted MK ploidy toward higher numbers (Fig. 5). Whereas the majority of glycoprotein Ib-positive cells had a DNA content less than 8N in cultures treated only with IL-3, the addition of GST-PLP-E resulted in a higher percentage of MK cells with a DNA content of 16N and 32N. The 16N/32N cell population accounts for 31% of all gated cells in the cultures treated with the combination of IL-3 and GST-PLP-E compared with only 18% in cultures treated with IL-3 alone.
Cytokines that induce hematopoietic differentiation are often capable of enhancing lineage-specific colony formation in semi-solid culture media, consistent with the results in Fig. 3. However, based on colony size, GST-PLP-E alone does not appear to promote proliferation of MK progenitors and instead seems to contain only differentiation-inducing activity, similar to the activities reported for IL-6 and IL-11 (20) and in contrast to the combined proliferative and differentiating activities reported for TPO (21). Compared with control treatments with IL-3 and GST or with IL-3 alone, colonies formed in the presence of IL-3 plus GST-PLP-E contained cells with much larger sizes and darker staining for AchE, indicative of a greater degree of differentiation induced by PLP-E (Fig. 6). In combination with IL-3, GST-PLP-E, IL-6, and TPO each stimulated an approximately 2-fold increase in CFU-MK number, and in the presence of IL-6 or TPO, GST-PLP-E treatment resulted in an additive effect on CFU-MK formation, suggesting that these factors may act through distinct molecular targets (Fig. 7). Taken together, these results suggest that PLP-E may not directly stimulate MK progenitor proliferation but may either enhance progenitor survival or induce multipotential progenitors to commit to the MK lineage.

Megakaryocytopoietic Activity of PLP-E Is Dependent on gp130—Several cytokines that induce MK cell differentiation act through heteromeric receptor complexes that include the signal transducing transmembrane protein, gp130 (20). To test the possibility that PLP-E also signals through gp130, monoclonal antibodies that block gp130 signaling were added along with GST-PLP-E to primary bone marrow cultures. These antibodies completely abolished MK differentiation in response to GST-PLP-E; colonies grown in the presence of GST-PLP-E and these antibodies contained MK cells of a small size similar to cultures treated with IL-3 alone (Fig. 8, A and C). In contrast, TPO-induced MK differentiation was unaffected by the addition of these antibodies (Fig. 8, D and E), consistent with TPO signaling through a distinct pathway. Furthermore, a blockade of gp130 signaling significantly reduced GST-PLP-E-induced colony formation, whereas gp130 antibodies had no detectable effect on TPO activity in this assay (Fig. 8F).
DISCUSSION

Mouse placental hormones related to PRL, now numbering more than a dozen, likely represent a group of cytokines that act on an array of targets in the mother to convert normal adult physiology to the physiology of pregnancy. As cytokines, primary targets for these hormones are expected to be found in the maternal hematopoietic system. Furthermore, direct effects of placental hormones on the hematopoietic system would be consistent with the large changes in blood volume and in blood cell development and function that are essential aspects of mammalian pregnancy. One important hematopoietic target for pregnancy-specific regulation is the MK lineage, which upon terminal differentiation gives rise to blood platelets, and our results have identified PLP-E as a pregnancy-specific inducer of MK cell differentiation.

The effective dose of PLP-E in the induction of MK cell differentiation is high relative to effective concentrations for many other cytokines. However, this is a common finding for placental hormones, which are typically produced in massive amounts, and the several µg/ml range is physiologically relevant for PLP-E. This activity cannot be attributed to a contaminant in the preparation because comparable preparations of GST alone or of a related GST fusion protein, GST-PLP-B, have no activity in these assays, because AP-PLP-E protein demonstrates specific binding of PLP-E to MK cells, and because the cytokine activity is recovered from bacterial cells, which are not producing any other mammalian factors.

PLP-E is most highly expressed on days 10–12 of gestation in the mouse (14), and therefore the effects of this hormone would largely be restricted to mid-gestation. However, our previous characterization of PLP-E included the identification and characterization of another placental-specific hormone, PLP-F, that is very similar in sequence to PLP-E; indeed, the sequence similarity between PLP-E and PLP-F is greater than between any other pair of hormones in this family thus far characterized in the mouse (14). PLP-F is expressed later in gestation, with high levels on days 15–16 (14). Preliminary results indicate that PLP-F also targets MK cells, and therefore this hormone

\[ J. \text{Lin, H. Lum, and D. Linzer, unpublished observations.} \]
may functionally replace PLP-E in late pregnancy. Thus, PLP-E and PLP-F may provide another example of what appears to be a common theme in placental endocrinology, namely a temporally programmed switch in the synthesis of two functionally related hormones during pregnancy. Synthesis of the two placental lactogens, one in early- to mid-pregnancy and the second in mid- to late-pregnancy, provided the first known example, and the early expression of the angiogenic hormone, prolferin, followed by the production of the angiogenic hormone, prolferin-related protein, represents a second example of such a temporal switch. It is not yet known if PLP-E and PLP-F have similar activities (as is seen for the two placental lactogens), in which case this pair of hormones may provide a means of maintaining the increase in MK differentiation throughout gestation; alternatively, PLP-E and PLP-F could conceivably have opposing effects (as is seen for prolferin and prolferin-related protein).

Analysis of the PLP-E amino acid sequence predicts that the hormone is glycosylated, and molecular forms of PLP-E larger than expected from the primary amino acid sequence alone are detected in maternal plasma. Glycosylation may contribute to the half-life of PLP-E in the maternal circulation, to the distribution of this hormone (for example, glycosylation may determine whether or not PLP-E can enter the fetal compartment), or to a maximal affinity for receptor, but this post-translational modification is not strictly required for receptor binding because the bacterial expressed PLP-E shows specific MK cell binding and is functional. Because antibodies to gp130 block PLP-E induction of MK cell differentiation, hormone function probably involves ligand interaction with a cell surface-binding protein followed by an interaction of the ligand-binding protein pair with gp130. The binding subunit may only recognize PLP-E, but this seems unlikely because PLP-E was also found to bind to adult male and adult, nonpregnant female MK cells, cells that would not be exposed to a placental-specific hormone. Several cytokines, including IL-6, IL-11, leukemia inhibitory factor, and ciliary neurotrophic factor, are megakaryocytopoietic and signal through gp130 (20), consistent with gp130 providing an important regulatory target for MK development and platelet production. Whether or not PLP-E shares receptor binding subunits with any of these other cytokines remains to be determined; possibly, PLP-E may prove to be a useful probe for identifying additional cytokine receptors on MK cells that may be therapeutically targeted for the clinical regulation of platelet production. Although it would be ideal to examine a requirement for PLP-E in megakaryocytopoiesis, for example by targeted disruption of the PLP-E gene, the existence of several other cytokines that also induce MK cell differentiation through a gp130 signaling pathway and that would likely provide compensatory activities makes this question difficult to address at this time.

Our investigation was directed at maternal targets of PLP-E, but it is also possible that this hormone enters the fetal compartment where it may contribute to fetal hematopoiesis at mid-gestation. In addition, although we only detected binding and activity of PLP-E on MK cells, we cannot exclude the possibility that PLP-E (perhaps in combination with other factors) may contribute to the differentiation of other hematopoietic lineages. Cytokines in the IL-6 subfamily, for example, have pleiotropic effects on multiple hematopoietic cell types (20). Preliminary experiments revealed that PLP-E is capable of inducing differentiation of mouse erythroleukemia cells into globin-expressing cells (22), and we have observed that PLP-E alone can induce colony formation in primary bone marrow cultures of non-MK lineages that we have not yet identified (data not shown). Thus, PLP-E may eventually be revealed as a multi-functional hormone in the regulation of pregnancy-specific hematopoiesis.

Acknowledgments—We thank Doug Engel for CD41 antibody, Mike Soares for the expression vector, and Tetsuya Taga for the anti-gp130 monoclonal antibodies. We also thank Doug Engel for many helpful comments and for critical reading of the manuscript. The expert technical assistance of Weimin Song and Diane Mayer is greatly appreciated.

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