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Decidual Cells Produce a Heparin-binding Prolactin Family Cytokine with Putative Intrauterine Regulatory Actions*

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Pregnancy in mice and rats is associated with the production of a large family of hormones/cytokines related to prolactin (PRL). The hormones/cytokines are hypothesized to coordinate maternal and fetal adaptations to pregnancy. In this study, PRL-like protein-J (PLP-J, also known as PRL family 3, subfamily c, member 1 (Prl3c1)) is shown to be a product of the uterine decidua and a regulator of postimplantation intrauterine events. PLP-J-specific antibodies and a series of recombinant PLP-J proteins were generated and used to investigate PLP-J expression and as ligands for investigating biological targets. Decidual PLP-J migrates as a 29-kDa protein and localizes to a band of decidual cells surrounding the trophoblast cell layer on gestation day 8.5. PLP-J ligands specifically bound *in situ* to the surrounding uterine stromal cells and vasculature within the decidua of gestation day 8.5 implantation sites. We then investigated the *in vitro* actions of PLP-J on uterine stromal cells and endothelial cells. PLP-J specifically interacted with both cell populations. PLP-J promoted uterine stromal cell proliferation and inhibited endothelial cell proliferation. We determined that PLP-J does not interact with PRL receptors. Instead, PLP-J interacts with heparin-containing molecules, including syndecan-1, which is expressed in gestation day 8.5 pregnant uteri, as well as in uterine stromal cells and endothelial cells. The restricted expression of PLP-J and its specific interactions with uterine stromal cells and endothelial cells suggests that it acts locally and regulates decidual cell development and the endometrial vasculature.

Successful pregnancy requires specialized maternal adaptations. Decidualization is a key uterine adaptation associated with the establishment of pregnancy and is characterized by the differentiation of uterine stromal cells (1–4). Decidual cell differentiation is dependent upon ovarian steroid hormone production, and in rodents, it also requires signals emanating from the preimplantation embryo (1, 5). Once formed, decidual cells establish a protective environment, facilitating the development of the placenta and embryo. They promote the redistribution of specific populations of leukocytes and reorganize the uterine vascular network. Intercellular signals elaborated by decidual cells are key mediators of these uterine adaptive responses. Among the decidual cell ligands are a family of cytokines related to prolactin (PRL).[2]

PRL is an ancient hormone with its origins as a regulator of vertebrate environmental adaptations (6, 7). Some species possess a single member of the PRL family that can be expressed in an assortment of tissues, including the anterior pituitary and uterus through the utilization of cell-specific promoters (8–10). Other species have undergone a gene expansion within the PRL locus (11). PRL family gene expansion is particularly robust in mice and rats (12–14). Gene duplication and natural selection have yielded 2 dozen related genes in each of these species. The PRL family genes encode cytokines/hormones that are expressed in cell-specific and temporally specific patterns and are most relevant to pregnancy-associated tissues, especially in the uterine decidua and the placenta. Initial observations suggest that the expanded PRL family participates in pregnancy-dependent adaptations to physiological stressors (15, 16). Although a few members are PRL mimetics (placental lactogens), activating PRL receptor signaling cascades, most utilize distinct strategies to regulate their cellular targets (11, 17). The cellular targets are intriguing and include endothelial cells, inflammatory/immune cells, and hematopoietic precursors (18–22).

Decidual cells of mice and rats express four members of the PRL family: PRL (23, 24), PRL-like protein-B (PLP-B; also known as PRL family 6, subfamily a, member 1 (Prl6a1)) (25–28), decidual PRL-related protein (dPRP; also known as PRL family 8, subfamily a, member 2 (Prl8a2)) (27, 29, 30), and PLP-J (also known as PRL family 3, subfamily c, member 1 (Prl3c1)) (31–34). PRL is postulated to be expressed in rodent decidual cells under the direction of a unique cell-specific promoter, as has been characterized for human decidual cells (10, 35), where it acts through the PRL receptor signaling pathway to promote decidual cell survival, regulate leukocyte function, stimulate uterine gland development, and facilitate vascular remodeling (23, 36–38). PLP-B and dPRP do not utilize the canonical PRL receptor signaling pathway (22, 26, 39). dPRP is a heparin binding cytokine that is essential for pregnancy-dependent adaptive responses to hypoxia (16, 22, 39). Unlike wild-type animals,

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[2] The abbreviations used are: PRL, prolactin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CHO, Chinese hamster ovary; NTA, nitrilotriacetic acid; AP, alkaline phosphatase; BSA, bovine serum albumin; FGF2, fetal growth factor 2; BrdUrd, bromodeoxyuridine.

mice deficient in dPRP do not effectively adapt to hypoxia and terminate their pregnancies. dPRP modulates decidual expression of PLP-J but not PLP-B or PRL (16). The expression of PLP-J is significantly decreased in dPRP null mice, suggesting that the biology of dPRP and PLP-J may be linked. Information on the biological functions of PLP-J is not available.

In this study, we have characterized the PLP-J protein and its expression pattern, identified targets for its action, and determined biological responses of its cellular targets. PLP-J is a heparin-binding cytokine with distinct actions on uterine stromal cell and endothelial cell populations.

EXPERIMENTAL PROCEDURES

Animals and Tissue Preparation

Holtzman rats were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN). The animals were housed in an environmentally controlled facility, with lights on from 0600 to 2000 h and were allowed free access to food and water. Timed pregnancies were generated, and tissue dissections were performed as previously detailed (40). Conceptuses with associated uteri were removed on specific days of gestation. Tissues were frozen in dry ice-cooled heptane and stored at −80 °C until used for *in situ* hybridization, immunohistochemistry, and *in situ* ligand binding or were frozen in liquid nitrogen and stored at −80 °C for subsequent RNA and protein analyses. The presence of sperm in the vaginal smear was designated as day 0.5 of pregnancy. New Zealand White rabbits were obtained from Myrtle's Rabbitry (Thompsons Station, TN) and used for antibody production. Protocols for the care and use of animals were approved by the University of Kansas Animal Care and Use Committee.

Cell Culture

U1 rat uterine stromal cells were obtained from Dr. Virginia Rider (Pittsburg State University, Pittsburg, KS) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, and penicillin (100 units/ml) and streptomycin (100 μg/ml) (41, 42). These rat uterine stromal cells are physiologically relevant in that they can be induced to differentiate into decidual cells (43). Rat aortic endothelial cells were purchased from VEC Technologies, Inc. (Rensselaer, NY) and maintained in MCDB-131 complete culture medium. The rat Nb2 lymphoma cell line was provided by Dr. Peter Gout (University of British Columbia, Vancouver, Canada) and maintained in RPMI 1640 culture medium supplemented with 10% horse serum, 10% FBS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 5 mM HEPES, penicillin (50 units/ml), and streptomycin (50 μg/ml) (44). Human embryonic kidney (HEK 293) cells were obtained from ATCC (Manassas, VA) and used as a host for the expression of the PLP-J fusion proteins. HEK 293 cells were maintained in DMEM/F-12 medium supplemented with 10% FBS, 1 mM sodium pyruvate, penicillin (100 units/ml), and streptomycin (100 μg/ml). CHO cells and heparan sulfate-deficient CHO-pgsD-677 cells were obtained from ATCC and cultured in DMEM/MCDB 302 culture medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Raji cells stably transfected with a syndecan-1 (Sdc1) expression vector (Raji-S1) and the parent Raji cell line were obtained from Dr. Alan C. Rapraeger (University of Wisconsin, Madison, WI) and were maintained in RPMI 1640 culture medium supplemented with 10% FBS and antibiotics (45). All cell cultures were maintained in a humidified atmosphere of 5% CO_2, 95% air at 37 °C.

Generation of Fusion Proteins

mFLAG-PLP-J—PLP-J was expressed as a fusion protein with a FLAG-His_6-FLAG tag. The full-length mature rat PLP-J cDNA was used as a template for PCR amplification of a PLP-J fragment with EcoRI and XbaI restriction sites at the 5′- and 3′-ends, respectively, using sequence specific primers, 5′-cat tta aag aat tca cac cat atg acc aga tgt-3′ and 5′-gtt ata tgt ttc tag att acc act tgt taa taa tg-3′. After digestion with EcoRI and XbaI restriction enzymes, the fragment was ligated into a modified pFLAG-CMV-3 vector (mFLAG; Sigma). The accuracy of vector construction was verified by DNA sequencing. The mFLAG-PLP-J plasmid was transfected into HEK 293 cells using Lipofectamine Plus according to the manufacturer's instructions (Invitrogen). The initial selection of transfected cells was accomplished in the presence of G418 at a concentration of 500 μg/ml. Selected cells were then maintained in 100 μg/ml G418.

The mFLAG-PLP-J fusion protein was purified from serum-free conditioned medium by incubating with an Ni^{2+}-NTA-agarose resin (Qiagen, Valencia, CA). In brief, Ni^{2+}-NTA-agarose was equilibrated with Sorensen's phosphate buffer (NaH_2PO_4 (66 mM) and KH_2PO_4 (66 mM)) and added to conditioned medium in a buffer containing 10 mM imidazole, 50 mM NaH_2PO_4, 0.15 M NaCl, pH 8.0 (1× binding buffer), incubated overnight at 4 °C with constant shaking. The resin was then transferred into a column and washed with 2× binding buffer. Recombinant mFLAG-PLP-J protein was eluted with 250 mM imidazole and 0.15 M NaCl in Sorensen's phosphate buffer, pH 6.0. Aliquots of fractions were separated using SDS-PAGE and stained with Coomassie Blue G-250 and immunoblotted with anti-FLAG M2 antibody (Sigma). Immunopositive fractions were pooled and dialyzed against phosphate-buffered saline, pH 7.4, and concentrated by Centricon ultrafiltration centrifugation devices (Millipore, Billerica, MA). Purified proteins were sterilized using Millex filters (Millipore), and concentrations were determined with the DC protein assay (Bio-Rad).

Alkaline Phosphatase (AP)-PLP-J Fusion Protein (AP-PLP-J)—The full-length cDNA for mature rat PLP-J was ligated downstream of heat-stable human placental AP of pCMV-SEAP and then transfected into HEK 293 cells, as previously described (46, 47). The transfected cells were selected and maintained in DMEM/F-12 culture medium supplemented with 10% FBS and G418 as described above. Cells were transferred to serum-free culture medium for 72 h. Conditioned medium was collected, and cellular debris was removed by centrifugation at 2,200 × *g* for 30 min at 4 °C and then stored at −20 °C. AP activity was quantified by a colorimetric assay at 405 nm using *p*-nitrophenyl phosphate as an AP substrate (47). AP fusion proteins were also separated using SDS-PAGE and electrophoretically transferred to nitrocellulose, and AP activity was detected by incubation with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Preparation of PLP-J Antibodies

Antibodies directed to PLP-J were prepared by immunizing rabbits with recombinant His_6-tagged PLP-J protein generated in a prokaryotic expression system, pQE-30Xa (Qiagen). The mature rat PLP-J cDNA was used as a template for PCR amplification of a PLP-J fragment with BamHI and SacI restriction sites at the 5′- and 3′-ends, respectively, using sequence specific primers, 5′-cat tta aag gat cca cac cat atg acc aga tgt-3′ and 5′-gtt ata tgt cga gct ctt acc act tgt ttt taa taa tg-3′. The amplified fragment was ligated into the pQE-30Xa plasmid and transformed into *Escherichia coli*, M15[pREP4]. His_6-PLP-J expression was induced by 2 mM isopropyl β-D-1-thiogalactopyranoside, and His_6-PLP-J protein was purified from bacterial exclusion bodies by Ni^{2+}-NTA-agarose affinity chromatography, as described above. Purified His_6-PLP-J was characterized by SDS-PAGE and Western blotting with anti-His tag antibodies (Qiagen). Purified His_6-PLP-J was used to immunize New Zealand White rabbits as previously described (48, 49).

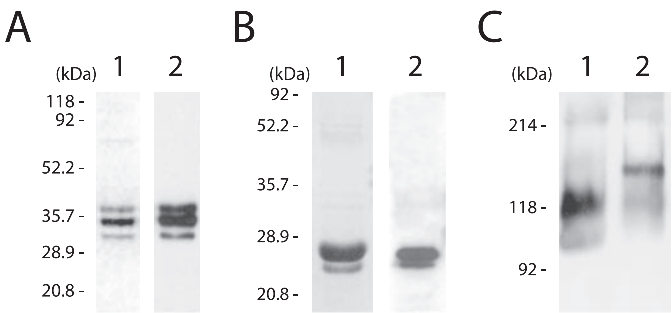


FIGURE 1. **SDS-polyacrylamide gel electrophoresis analysis of purified PLP-J fusion proteins.** mFLAG-PLP-J (*A*) and His-PLP-J proteins (*B*) were purified with Ni^{2+}-NTA-agarose affinity chromatography and resolved in 12% SDS-polyacrylamide gels under reducing conditions. Total protein in the preparations was detected by Coomassie Blue staining (*lane 1* of *A* and *B*), and PLP-J-containing proteins were detected by Western blotting with anti-PLP-J (*lane 2* of *A* and *B*). *C*, culture media conditioned by HEK 293 cells transfected with a CMV-driven human placental AP (*lane 1*) plasmid or a CMV-driven AP-PLP-J fusion gene (*lane 2*) were separated by SDS-PAGE, blotted to nitrocellulose membrane, and analyzed for AP activity by staining with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Western Blot Analysis of Decidual Tissues

Protein lysates were prepared by homogenizing gestation day 8.5 rat conceptuses (decidua and extraembryonic and embryonic tissues) in radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.2, 1% Triton X-100 or 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). Protein concentrations were determined by the DC protein assay (Bio-Rad). Fifty μg of total protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Immunoreactive PLP-J and dPRP were detected with antibodies to anti-PLP-J (present study) and anti-dPRP (39), respectively, and visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

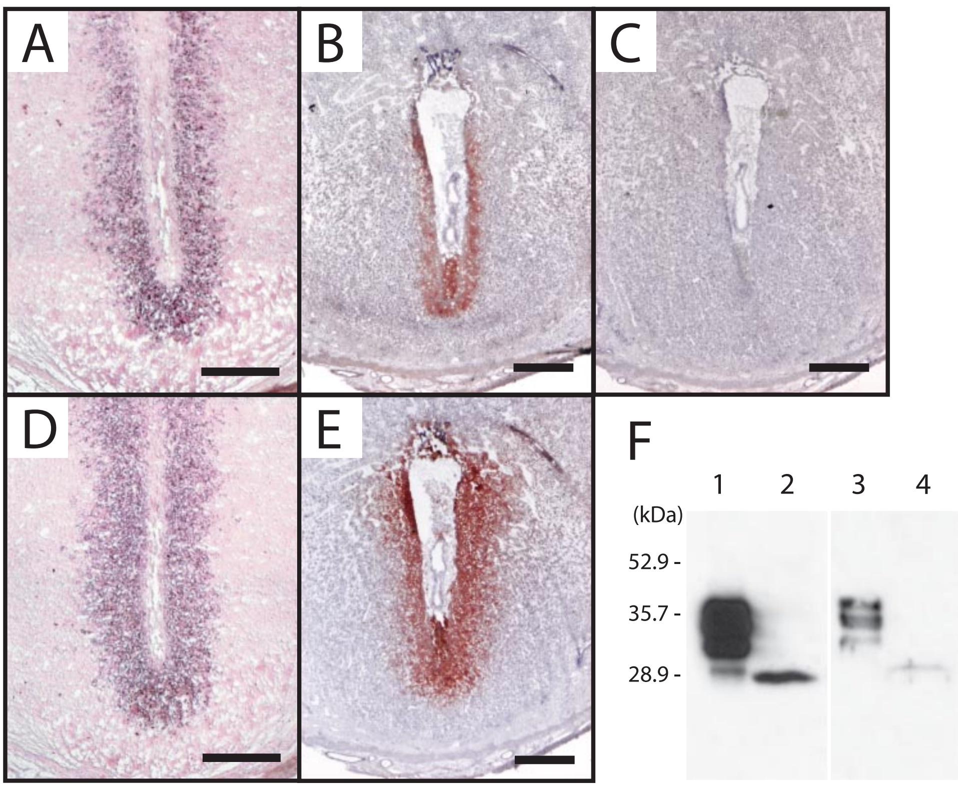
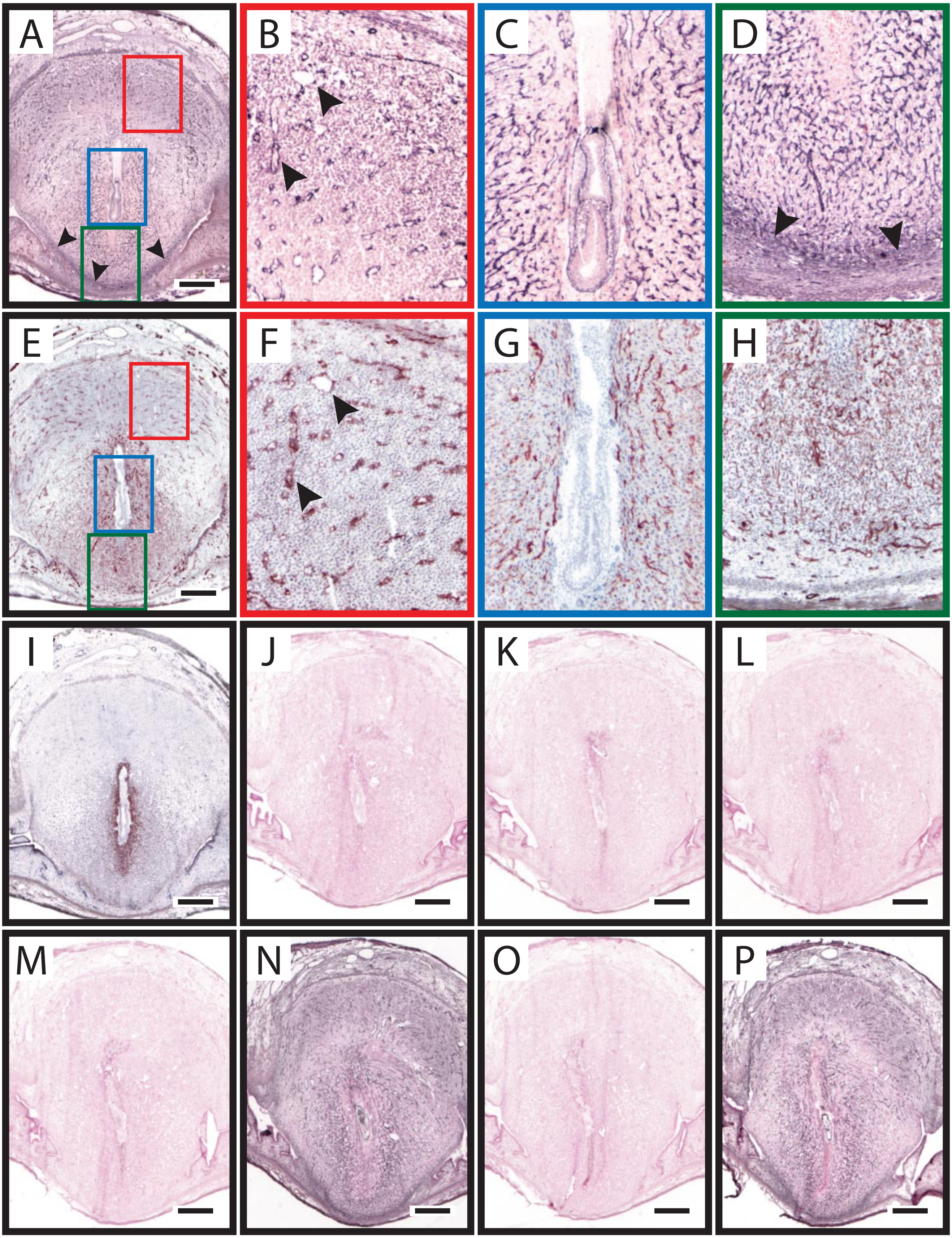


FIGURE 2. **Expression of PLP-J in day 8.5 rat implantation sites.** PLP-J expression was detected in cryosections from gestation day 8.5 rat implantation sites by *in situ* hybridization (*A*) and immunostaining (*B*). The specificity of the immunoreactivity was determined by competing with purified mFLAG-PLP-J (*C*). PLP-J mRNA and protein were localized to decidual cells surrounding the developing embryo in distributions similar to the expression patterns of dPRP mRNA (*D*) and protein (*E*). *Scale bars* in *A–E*, 500 μm. *F*, detection of PLP-J in gestation day 8.5 rat decidual extracts. Extracts were separated in 12% SDS-polyacrylamide gels, and PLP-J was detected by Western blotting using anti-PLP-J. *Lanes 1* and *3*, purified mFLAG-PLP-J; *lanes 2* and *4*, gestation day 8.5 rat decidual extracts. *Lanes 3* and *4*, anti-PLP-J was co-incubated with purified mFLAG-PLP-J.

Immunocytochemistry

Immunocytochemical analyses were used to localize PLP-J and dPRP proteins and the distribution of endothelial cells in gestation day 8.5 implantation sites, as described (39, 40, 50). Cryosections (10 μm) were prepared, fixed in cold 4% paraformaldehyde solution, and blocked in 10% normal goat serum for 1 h at room temperature. The immunodetection was performed by incubating overnight at 4 °C with anti-PLP-J (present study), anti-dPRP antibodies (39), or RECA-1 antibodies, which recognize an uncharacterized rat endothelial cell-specific surface antigen (Serotec, Oxford, UK) (41). Avidin-peroxidase-conjugated secondary antibody was added for 30 min at room temperature and color-developed with an AEC kit (Zymed Laboratories, San Francisco, CA). Tissues were counterstained with Mayer's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hematoxylin. Images were captured using a Leica MZFIII steromicroscope (Leica Microsystems GmbH, Welzlar, Germany) or a Nikon Eclipse 55i microscope (Nikon Instruments Inc., Melville, NY), both equipped with Leica CCD cameras (Leica).

In Situ Hybridization

In situ hybridization was performed to assess the distributions of PLP-J and dPRP transcripts in gestation day 8.5 rat implantation sites (40, 51). Cryosections (10-μm) were prepared and stored at −80 °C until used. Plasmids containing cDNAs for PLP-J (34) and dPRP (29) were used as templates to synthesize sense and antisense digoxigenin-labeled riboprobes according to the manufacturer's instructions (Roche Applied Science). The frozen sections were air-dried and fixed in cold 4% paraformaldehyde in phosphate-buffered saline. Prehybridization, hybridization, and detection of alkaline phosphatase-conjugated anti-digoxigenin were performed as previously reported (12, 51). Images were captured as described above.

Northern Blot Analysis

Northern blot analysis was performed as previously described (52). Total RNA was extracted from gestation day 8.5 rat decidual tissues, U1 uterine stromal cells, and rat aortic endothelial cells using TRIzol reagent (Invitrogen). Total RNA (15 μg/lane) was resolved in 1% formaldehyde-agarose gels, transferred to nylon membranes, and cross-linked. Blots were probed with ^{32}P-labeled cDNAs for syndecans (*Sdc1*, NM_013026; *Sdc2*, NM_013082; *Sdc3*, NM_053893; *Sdc4*, NM_012649). Glyceraldehyde-3-phosphate dehydrogenase cDNA was used to evaluate the integrity and equal loading of RNA samples. At least three different tissue samples from three different animals were analyzed with each probe for each time point.

AP-PLP-J Binding

Tissues—An *in situ* AP-binding assay was performed as previously described (46, 47). In brief, 8–10-μm tissue sections were prepared with a cryostat and mounted onto glass slides. The tissue sections were washed with a modified Hanks' balanced salt solution (HBHA; containing 20 mM HEPES, 0.5 mg/ml BSA and 0.1% NaN3) and incubated with AP, AP-PLP-J, or AP-placental lactogen-I (PL-I; also known as PRL family 3, subfamily d, member 1 (Prl3d1)) fusion protein (46) for 75 min at room temperature. For competition, tissue sections were incubated with various glycosaminoglycans and/or mFLAG-PLP-J protein for 45 min prior to incubation with AP-PLP-J. Following incubation, tissue sections were washed three times with HBHA containing 0.1% Tween 20 and fixed for 20 min with acetone-formaldehyde fixative. The fixed sections were washed and heated at 65 °C for 30 min to inactivate endogenous AP activity in the tissues. Localization of AP was determined by incubation with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Cells—For AP-ligand binding with cells, 5 × 10^4 cells were incubated with different concentrations of conditioned medium of AP or AP-PLP-J for 60 min at room temperature. Where indicated, cells were incubated with different concentrations of heparin for 30 min before incubation with AP-PLP-J. Cells were washed with HBHA containing 0.1% Tween 20 and HBSS. Cells were then heated for 30 min at 65 °C to inactivate endogenous AP activity. AP activity was determined by incubation with AP substrate (*p*-nitrophenyl phosphate) and measurement of absorbance at 405 nm. AP-PLP-J binding to heparan sulfate-deficient CHO-pgsD-677 cells and wild-type CHO cells was also assessed as previously described (22). AP-PLP-J and AP-PL-I binding with the PRL receptor were assessed in CHO-pgsD-677 cells (53) transiently transfected with the PRL receptor (pECE/long; gift from Dr. Paul Kelly, INSERM, Paris, France) (54) using FusGene 6 (Roche Applied Science). The AP-PL-I fusion protein was used as a positive control.

Heparin Binding Assay

PLP-J and dPRP interactions with heparin were evaluated with a heparin-binding plate assay (55). Multiwell plates were coated with either heparin conjugated to bovine serum albumin (heparin-BSA; Sigma) or BSA (Sigma) and incubated with AP-PLP-J, AP-dPRP, or AP. Wells were washed with HBHA containing 0.1% Tween 20 and HBSS. AP activity was determined by incubation with AP substrate (*p*-nitrophenyl phosphate) and measurement of absorbance at 405 nm.

Cell Adhesion Assay

Cell adhesion assays were performed as described (56). 96-well microtiter plates were coated with test proteins diluted in PBS (50 μl/well) and incubated at 4 °C for 16 h. Wells were washed three times with PBS and blocked with 1% BSA at room temperature for 1 h. To test for specificity, anti-PLP-J serum or normal rabbit serum was added to the wells and incubated for 1 h at 37 °C prior to plating of cells. Cells were harvested in normal growth medium, washed with HBSS, and resuspended at 5 × 10^5 cells/ml in serum-free normal growth medium. Where indicated, cells were incubated with heparin prior to plating for 1 h at room temperature. A 50-μl cell suspension was added to each well and incubated 30 min at 37 °C. Adherent cells were fixed in 10% formalin in saline for 30 min at room temperature. The cells were stained with methylene blue, and adhesion was quantified by dye extraction and measurement of absorbance at 620 nm (56).

FIGURE 3. **PLP-J *in situ* binding within gestation day 8.5 rat implantation sites.** *A*, PLP-J binding to gestation day 8.5 implantation sites as detected by AP-PLP-J *in situ* binding. *B–D*, higher magnification images of the *boxes* indicated in *A*, corresponding to the mesometrial (*red box*), periembryonic (*blue box*), and antimesometrial regions (*green box*), respectively. The *arrowheads* in *A* and *D* show the location of PLP-J binding to the undifferentiated uterine stromal cell compartment. *E*, distribution of endothelial cells within gestation day 8.5 rat implantation sites shown by immunocytochemistry using an endothelial cell-specific antibody, RECA-1. *F–H*, higher magnification images of the *boxes* indicated in *E*, corresponding to the mesometrial (*red box*), periembryonic (*blue box*), and anti-mesometrial regions (*green box*), respectively. The *arrowheads* in *B* and *F* indicate examples of blood vessels that are positive for PLP-J binding and RECA-1 immunoreactivity. *I*, distribution of PLP-J protein expression within the gestation day 8.5 rat implantation site. *Panels J–L* demonstrate the specificity of AP-PLP-J binding to gestation day 8.5 rat implantation sites. *J*, AP binding; *K*, AP-PLP-J + mFLAG-PLP-J binding; *L*, AP-PLP-J + heparin binding; *M*, AP-PLP-J plus heparan sulfate binding; *N*, AP-PLP-J plus chondroitin sulfate A binding; *O*, AP-PLP-J plus dermatan sulfate binding; *P*, AP-PLP-J plus chondroitin sulfate C binding. Competition with all glycosaminoglycans was at a concentration of 50 μg/ml. *Scale bars* for *A*, *E*, and *I–P*, 1 mm.

Nb2 Lymphoma Cell Proliferation Assay

The Nb2 lymphoma cell proliferation assay was performed with some modifications to the previously published procedure (44). Twenty-four h before initiating the assays, cells were incubated with RPMI1640 supplemented with 5% horse serum, 2 mM L-glutamine, 5 mM HEPES, and antibiotics (Assay Medium) to establish a quiescent state. Cells were washed with Assay Medium, counted with a hemocytometer, and distributed to wells in a 96-well plate (2.5×10^4 cells/well). Cells were incubated with ovine PRL (0.1, 1, or 10 ng/ml; NOBL Laboratories, Inc., Sioux Center, IA) or mFLAG-PLP-J (0.1, 1, or 10 μg/ml) for 72 h. Viable cells were quantified by the CellTiter 96 AQ_{uous} nonradioactive cell proliferation assay (Promega, Madison, WI).

Rat Uterine Stromal Cell Proliferation Assay

Rat U1 uterine stromal cells were harvested by trypsin-EDTA, washed, and resuspended in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, and 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were distributed in 96-well plates at a density of 1×10^3 cells/well and incubated for 24 h. To initiate the assays, cells were starved for 24 h with DMEM medium containing 1% FBS. Cells were then treated with mFLAG-PLP-J (0.01, 0.1, 1, or 10 μg/ml) or fibroblast growth factor 2 (FGF2; 10 ng/ml; R&D Systems, Minneapolis, MN) for 24, 48, or 72 h. Numbers of viable cells were measured by the CellTiter 96 AQ_{uous} nonradioactive cell proliferation assay (Promega) at the indicated times. To assess DNA synthesis, cells were grown as described above and distributed in 24-well plates. Bromodeoxyuridine (BrdUrd; Sigma) was added to the medium (10 μM) for 16 h in the presence or absence of mFLAG-PLP-J. The BrdUrd incorporation was detected using a BrdUrd staining kit (BD Pharmingen).

Endothelial Cell Proliferation Assay

Rat aortic endothelial cells were harvested by trypsin-EDTA, washed, and resuspended in complete MCDB-131 medium. The cells were distributed in 48-well plates at a density of 4×10^3 cells/well and incubated for 24 h. To initiate the experiments, cells were incubated with MCDB-131 basal medium with 1% FBS for another 24 h. Cells were then treated with different concentrations of mFLAG-PLP-J (0.01, 0.1, 1, or 10 μg/ml) or FGF2 (10 ng/ml) for 24, 48, and 72 h. The numbers of viable cells were measured using the CellTiter 96 AQ_{uous} nonradioactive cell proliferation assay (Promega). The effects of PLP-J on endothelial cell DNA synthesis were determined with the BrdUrd incorporation assay as described above.

Cell Proliferation Monitored by Crystal Violet Staining

Proliferation indices of U1 rat uterine stromal cells and rat aortic endothelial cells were also evaluated morphologically by crystal violet staining, as previously described (57, 58). Cells were harvested with trypsin-EDTA, washed, resuspended at 5×10^4 cells/ml in growth medium, distributed in 48-well plates, and cultured for 24 h. Cells were washed and preincubated with medium containing 1% FBS for 24 h. Test proteins were then added to the cells. At the indicated time intervals, cells were stained with 0.1% crystal violet for 10 min, washed with distilled water, and dried at room temperature. Images of cells were captured with a Leica MZFIII stereomicroscope equipped with a CCD camera (Leica).

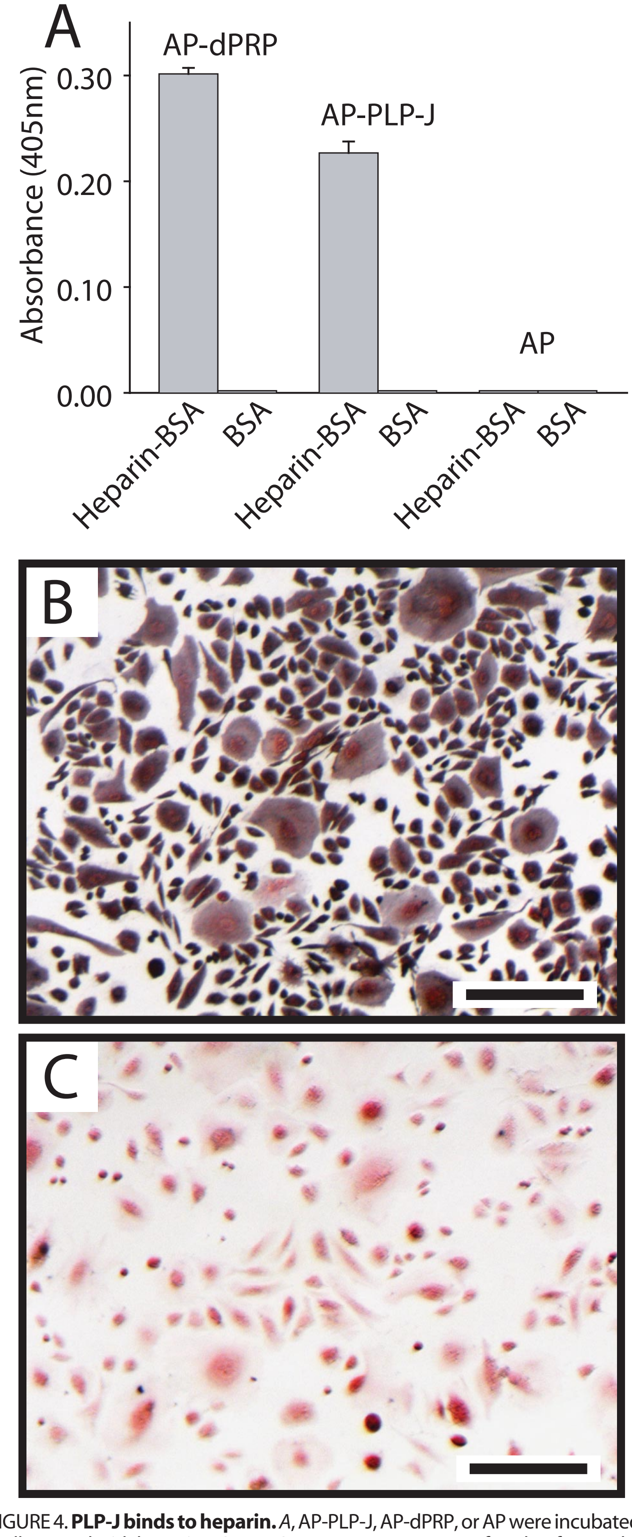

FIGURE 4. **PLP-J binds to heparin.** *A*, AP-PLP-J, AP-dPRP, or AP were incubated in wells coated with heparin-BSA or BSA at room temperature for 2 h. After washing with PBS and Tween 20, a substrate for AP (*p*-nitrophenyl phosphate) was added, and AP activity was measured by a colorimetric assay at 405 nm. *B* and *C*, AP-PLP-J interaction with wild type CHO cells (*B*) and heparan sulfate-deficient CHO-pgsD-677 cells (*C*). Please note that PLP-J specifically interacts with heparin-containing molecules. *Scale bars* in *B* and *C*, 250 μm.

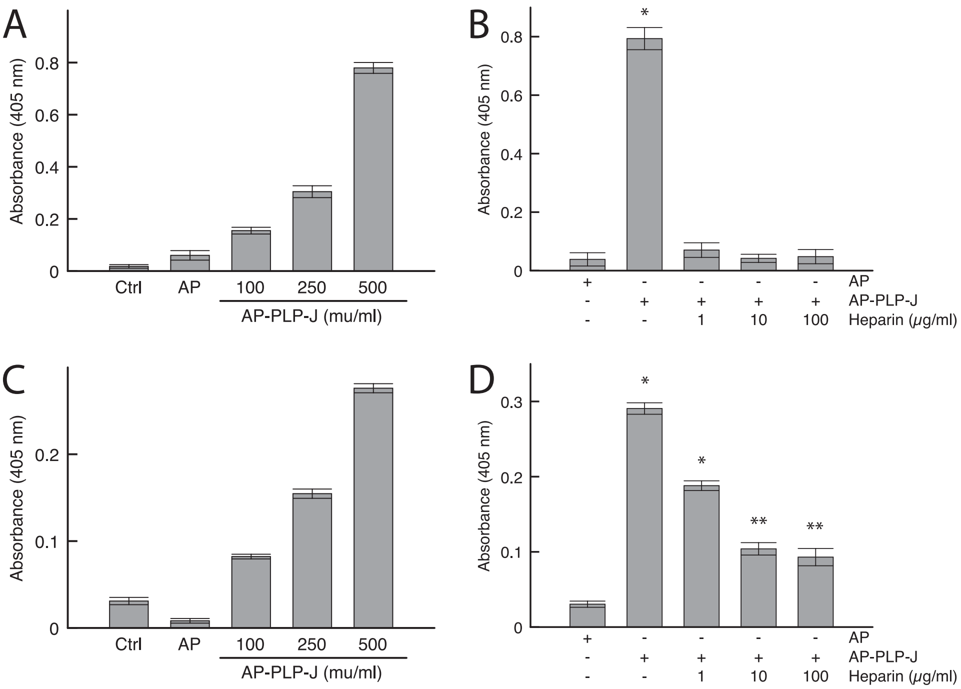

FIGURE 5. **PLP-J interactions with uterine stromal cells and endothelial cells.** Uterine stromal cells (*A* and *B*) and endothelial cells (*C* and *D*) were incubated with various concentrations of AP-PLP-J (*A* and *C*) or with AP-PLP-J with various concentrations of heparin (*B* and *D*) at room temperature for 75 min. After the incubation, cells were washed, and the endogenous AP inactivated by heating at 65 °C for 20 min. AP substrate, *p*-nitrophenyl phosphate, was added, and AP activity was measured as absorbance at 405 nm. Note that heparin was able to effectively decrease PLP-J interactions with both cell populations. However, heparin was not as efficient in competing for PLP-J interactions with endothelial cells. Values represent the mean ± S.E. of five replicates and are representative of three independent experiments. *Single asterisks* indicate significant differences from AP controls ($p < 0.01$), and *double asterisks* indicate significant differences from AP and AP-PLP-J controls; $p < 0.01$.

Statistical Analysis

Statistical comparisons between two means were determined with Student's *t* test. Comparisons among multiple means were evaluated with analysis of variance. The source of variation from significant *F*-ratios was determined with Bonferroni's multiple comparison test (59).

RESULTS

Generation of PLP-J Fusion Proteins and Antibodies—PLP-J was engineered to be expressed in recombinant form containing FLAG and His_6 tags at its N terminus (mFLAG-PLP-J) in HEK 293 cells. Purified mFLAG-PLP-J consisted of three species with molecular masses ranging from ~31 to 37 kDa (Fig. 1*A*). PLP-J possesses two putative *N*-linked glycosylation sites (31–34); thus, differential glycosylation may account for the mFLAG-PLP-J size variants. mFLAG-PLP-J was used as a ligand to evaluate PLP-J cellular actions (see below). PLP-J was also expressed as a His_6-tagged fusion protein in a bacterial expression system. The His_6-tagged PLP-J fusion protein migrated predominantly as a 26-kDa protein (Fig. 1*B*) and was used as an immunogen in rabbits. PLP-J antibodies reacted with both the mFLAG-PLP-J and His_6-tagged PLP-J fusion proteins (Fig. 1, *A* and *B*). AP and AP-PLP-J fusion proteins were generated in HEK 293 cells (Fig. 1*C*) and used as probes to assess PLP-J interactions with putative cellular and molecular targets.

Intrauterine PLP-J Expression and Binding—PLP-J expression patterns and PLP-J targets were investigated. The distributions of PLP-J and dPRP mRNAs within the uterus of pregnant rats were assessed by *in situ* hybridization and were consistent with earlier reports (29, 30, 32, 34). PLP-J and dPRP mRNAs were predominantly observed in the uterine decidua, especially within the antimesometrial decidua (Fig. 2, *A* and *D*). PLP-J and dPRP protein immunolocalizations mirrored the distribution of PLP-J and dPRP mRNAs (Fig. 2, *B* and *E*). Decidual PLP-J migrated as a single 29-kDa protein and thus differed from the multiple molecular species of recombinant HEK 293 cell-engineered mFLAG-PLP-J (Fig. 2*F*). The specificity of the PLP-J immunoreactivity was demonstrated by competition with purified mFLAG-PLP-J (Fig. 2, *C* and *F*, *lanes 2* and *4*). PLP-J interactions with structures within gestation day 8.5 implantation sites were also investigated (Fig. 3). AP-PLP-J binding was detected throughout the uterus and was particularly evident in association with the uterine vasculature (Fig. 3, *B*–*D* and *F*–*H*) and the undifferentiated uterine stromal cell compartment (Fig. 3*D*). Intrauterine distributions of AP-PLP-J binding and RECA-1 immunoreactivity (a rat endothelial cell-specific antibody) overlapped. Intrauterine AP-PLP-J binding was specific (Fig. 3, *J* and *K*) and could be competed by mFLAG-PLP-J (Fig. 3*K*), heparin (Fig. 3*L*), heparan sulfate (Fig. 3*M*), and dermatan sulfate (Fig. 3*O*) but not chondroitin sulfate A or C (Fig. 3, *N* and *P*). In summary, PLP-J protein is synthesized by uterine decidual cells situated proximal to the developing embryo and specifically interacts with intrauterine structures, especially the vasculature and undifferentiated uterine stromal cells, in a heparin/heparan sulfate- and/or dermatan sulfate-dependent manner.

PLP-J Interacts with Heparin—Based on the heparin-dependence of the AP-PLP-J-uterine tissue binding, we further investigated PLP-J-heparin interactions. dPRP, another decidual cell cytokine, also specifically interacts with heparin (22, 39) and was used as a positive control in some of the analyses. AP-PLP-J and AP-dPRP specifically bound heparin-BSA-coated plates but not BSA-coated plates, whereas AP did not bind to either plate (Fig. 4*A*). AP-PLP-J also exhibited binding to CHO cells that was dependent upon the presence of heparan sulfate (Fig. 4, *B* and *C*). Additional PLP-J-cellular interactions that are potentially physiologically relevant (uterine stromal cells and endothelial cells; see Fig. 3) were assessed. AP-PLP-J specifically interacted with both uterine stromal cells (Fig. 5*A*) and endothelial cells (Fig. 5*C*). Heparin was an effective competitor of AP-PLP-J binding to uterine stromal cells (Fig. 5*B*) but was less effective in competing with AP-PLP-J binding to endothelial

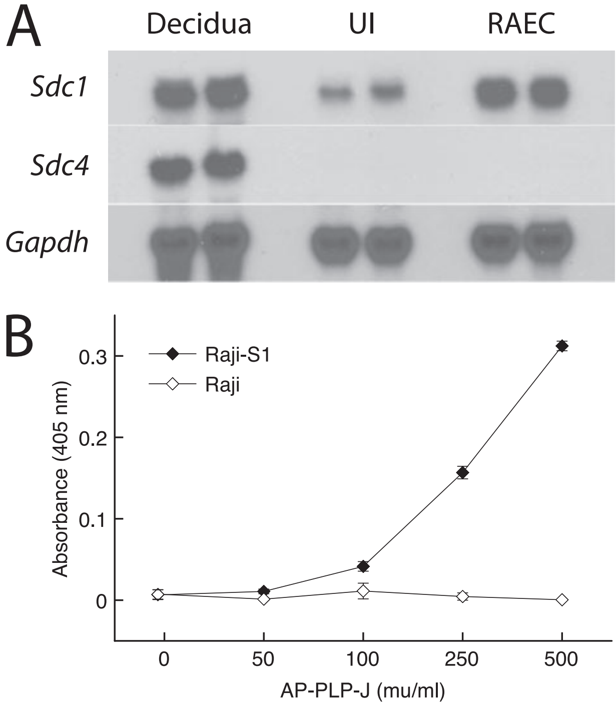

FIGURE 6. **PLP-J interactions with syndecans.** *A*, Northern blot analyses for *Sdc1* and *Sdc4*. Total RNA was isolated from gestation day 8.5 rat decidual tissues, U1 uterine stromal cells, and endothelial cells. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used to demonstrate integrity of the RNA and loading accuracy. *B*, AP-PLP-J binding to Raji cells and Raji cells transfected with a eukaryotic expression vector containing *Sdc1* (Raji-S1). Raji and Raji-S1 cells were incubated with AP-PLP-J. After washing, a substrate for AP (*p*-nitrophenyl phosphate) was added, and AP activity was measured by colorimetric assay at 405 nm. Values represent the mean ± S.E. of five replicates.

cells (Fig. 5*D*). Collectively, the results indicate that PLP-J associates with heparin-containing molecules, potentially located in extracellular matrices and on cell surfaces.

PLP-J Interacts with Syndecans—Syndecans are a family of four transmembrane proteins possessing heparan sulfate sugar chains associated with their extracellular domains (60, 61). They have been implicated in cell adhesion and growth factor signal transduction and were considered molecular candidates for PLP-J interaction with its target cells. Initially, uterine decidua, uterine stromal cells, and endothelial cells were assessed for their expression of syndecan family members by Northern blotting (Fig. 6*A*). Gestation day 8.5 uterine decidua expressed *Sdc1* and *Sdc4* mRNAs, whereas uterine stromal cells and endothelial cells expressed only *Sdc1* mRNA. *Sdc2* and *Sdc3* were not detected in decidua and the two cell populations. Raji cells are devoid of heparan containing molecules on their surfaces and represent a useful cell model for testing the biology of specific heparan sulfate proteoglycans (45). The ability of PLP-J to bind syndecans was evaluated in Raji cells genetically engineered to express *Sdc1* (45). AP-PLP-J interactions with Raji cells were shown to be dependent upon the surface expression of *Sdc1* (Fig. 6*B*). Thus, syndecans are expressed in the uterine decidua and are potential mediators of PLP-J-cellular interactions.

PLP-J Does Not Activate the Canonical PRL Receptor Signaling Pathway—A subset of members of the PRL family influence target cell function through activation of the PRL receptor signaling pathway (11). This subset includes PRL, PL-I, and PL-II (also known as PRL family 3, subfamily b, member 1 (Prl3b1)). Interestingly, PLP-J is also a member of the PL-I and PL-II subfamily. Its interactions with the PRL receptor signaling pathway

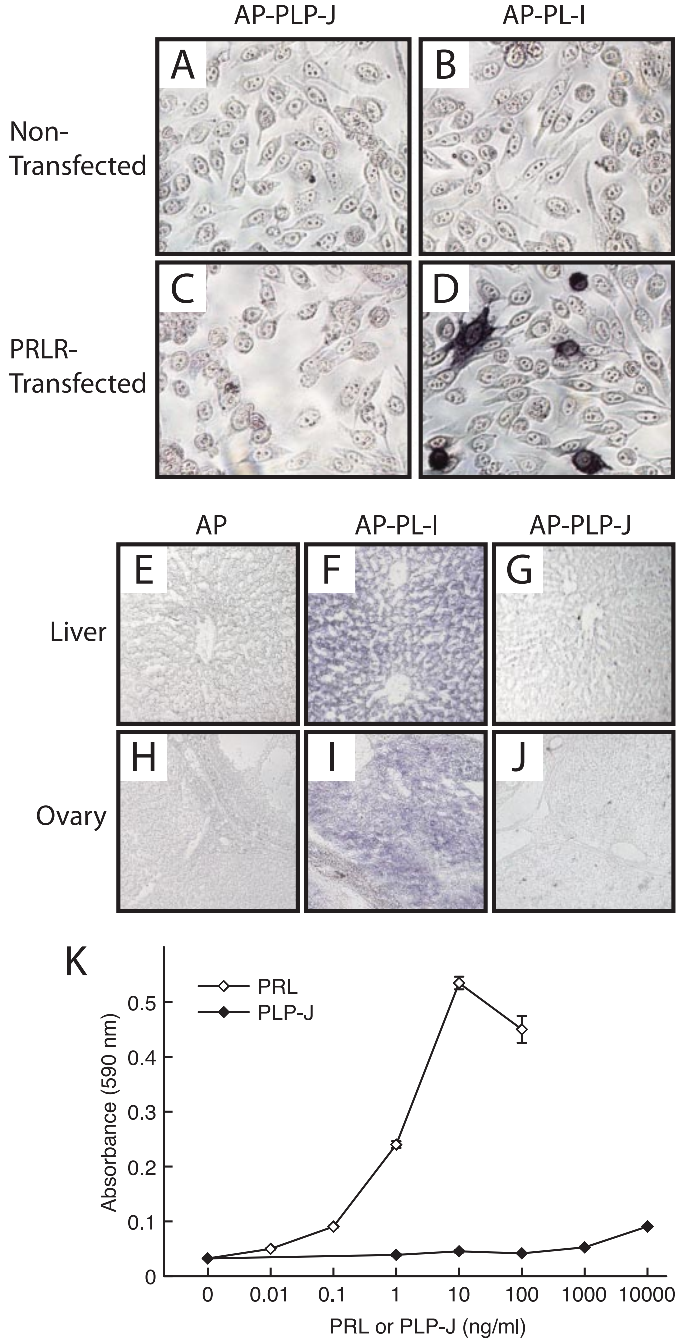

FIGURE 7. **PLP-J does not interact with or activate the PRL receptor.** CHO-pgsD-677 cells (heparan sulfate-deficient) were transiently transfected with the long form of the rat PRL receptor (pECE/long) and probed with AP-PLP-J (*A* and *C*) or AP-PL-I (*B* and *D*) fusion proteins. AP-PLP-J failed to bind to CHO-pgsD-677 cells transiently transfected with the long form of the rat PRL receptor. *E–J*, interactions of AP, AP-PL-I, and AP-PLP-J with tissues possessing abundant PRL receptors (gestation day 11.5 liver and ovary). AP-PLP-J binding was performed in the presence of excess heparin. AP-PL-I effectively bound to both liver and ovarian tissues, but AP and AP-PLP-J failed to bind. *K*, effects of PLP-J on Nb2 cell proliferation. Quiescent Nb2 cells were incubated with a range of concentrations of PLP-J or ovine PRL (positive control) for 72 h. Cell numbers were assessed with a nonradioactive cell proliferation assay. Values represent the mean ± S.E. of five replicates. Please note that PLP-J failed to stimulate the proliferation of Nb2 cells.

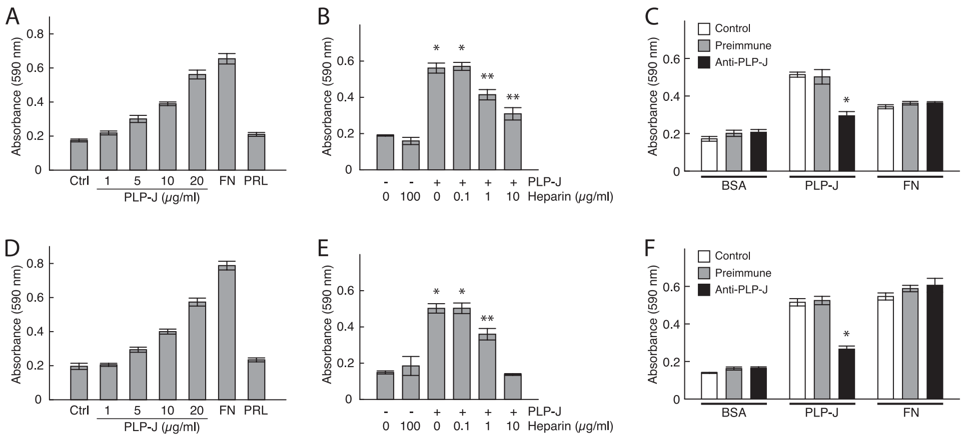

FIGURE 8. **Adhesion of uterine stromal cells and endothelial cells to PLP-J.** U1 uterine stromal cells (*A–C*) and endothelial cells (*D–F*) were assessed for their ability to bind to PLP-J. *A* and *D*, cells were plated in 96-well plates coated with BSA (*Ctrl*; 10 μg/ml), PLP-J (1–20 μg/ml), fibronectin (*FN*; 2.5 μg/ml), or ovine PRL (20 μg/ml). *B* and *E*, cells were plated in 96-well-plates coated with BSA (10 μg/ml), PLP-J (20 μg/ml), and various concentrations of heparin. *C* and *F*, cells were plated in 96-well plates coated with BSA (10 μg/ml), PLP-J (20 μg/ml), or fibronectin (2.5 μg/ml). In *C* and *F*, some wells were preincubated with preimmune serum or antiserum to PLP-J. For all experiments, cells were incubated at 37 °C for 30 min and washed, and adherent cells were fixed and stained with methylene blue. The dye was extracted from the adherent cells and quantified by absorbance at 600 nm. All values represent the mean ± S.E. of five replicates and are representative of three independent experiments. *Single asterisks* indicate significant differences from BSA controls ($p < 0.01$), and *double asterisks* indicate significant differences from BSA and PLP-J controls ($p < 0.01$).

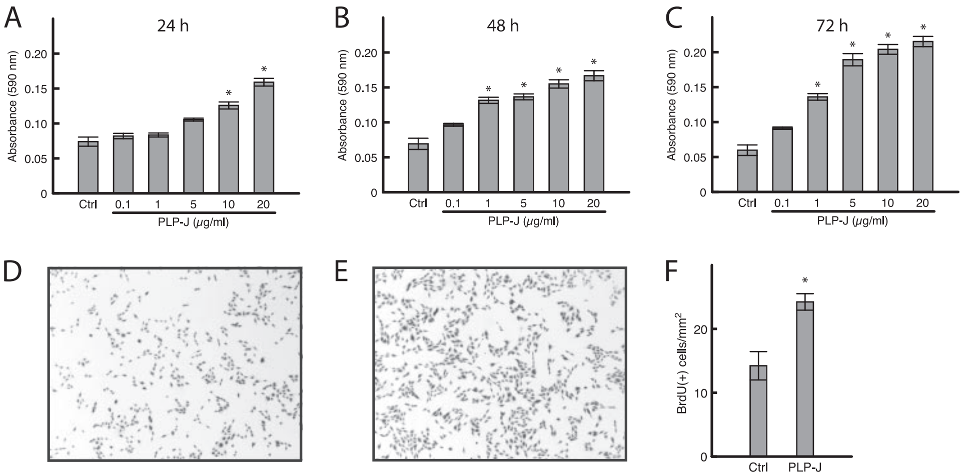

FIGURE 9. **PLP-J stimulates uterine stromal cell proliferation.** U1 uterine stromal cell proliferative responses to PLP-J were assessed by a nonradioactive cell proliferation assay (*A–C*), crystal violet staining (*D* and *E*), and BrdUrd incorporation (*F*). Proliferative responses were evaluated at 24 h (*A*), 48 h (*B*), and 72 h (*C*) and expressed as absorbance at 590 nm. Values represent the mean ± S.E. of five replicates and are representative of three independent experiments. *D* and *E*, U1 uterine stromal cells were treated with or without PLP-J (10 μg/ml) for 72 h and stained with 0.1% crystal violet. After washing, images were captured. *F*, effects of PLP-J (5 μg/ml) on U1 uterine stromal cell incorporation of BrdUrd. Cells were incubated in the presence of BrdUrd with or without PLP-J for 16 h. BrdUrd-labeled cells were identified with a BrdUrd detection kit (BD Pharmingen). Results are expressed as the percentage of BrdUrd-stained cells *versus* the total number cells. All values represent the mean ± S.E. of five replicates. *Asterisks* indicate significant differences from controls ($p < 0.01$).

were evaluated in the next series of experiments. Initially, binding experiments were performed with heparan sulfate-deficient CHO-pgsD-677 cells transfected with an expression vector containing the long form of the rat PRL receptor. AP-PL-I effectively interacted with the engineered cells, whereas AP-PLP-J was ineffective (Fig. 7, *A–D*). AP-PLP-J was also ineffective in binding to tissue sections of the rat corpus luteum and liver, tissues with abundant PRL receptor expression (Fig. 7, *E–J*). Rat Nb2 lymphoma cell proliferation has been used as a bioassay for canonical PRL receptor activation (44). PRL stimulated Nb2 lymphoma cell proliferation; however, PLP-J was ineffective (Fig. 7*K*). The results indicate that PLP-J is not a PRL receptor agonist.

PLP-J Promotes Uterine Stromal Cell and Endothelial Cell Adhesion—Since PLP-J interacts with heparin-containing molecules and heparin-related molecules are located on the cell surface of uterine stromal cells and endothelial cells, we next assessed the effectiveness of PLP-J in promoting uterine stromal cell and endothelial cell adhesion. In these experiments, fibronectin was used as a positive control and PRL as a negative control. PLP-J and fibronectin facilitated both uterine stromal cell and endothelial cell adhesion, whereas PRL was ineffective (Fig. 8, *A* and *D*). Co-incubation with heparin decreased cellular adhesion to PLP-J. Heparin was more potent in interfering with PLP-J-mediated endothelial cell adhesion (Fig. 8*E*) than with PLP-J-mediated uterine stromal cell adhesion (Fig. 8*B*). PLP-J-dependent adhesion was also disrupted by co-incubation with PLP-J antibodies (Fig. 8, *C* and *F*). The same antibodies did not interfere with fibronectin-mediated cellular adhesion. The findings indicate that PLP-J can act as an adhesion molecule for both uterine stromal cells and endothelial cells.

PLP-J Modulation of Uterine Stromal Cell and Endothelial Cell Proliferation—Uterine stromal cells and endothelial cells undergo dynamic changes during the establishment of pregnancy (1–4). The effects of PLP-J in modulating uterine stromal cell and endothelial cell proliferation were investigated with three complementary assays (Figs. 9 and 10). PLP-J stimulated uterine stromal cell proliferation (Fig. 9, *A–E*) and uterine stromal cell incorporation of BrdUrd (Fig. 9*F*). In contrast, PLP-J inhibited endothelial cell proliferation and endothelial cell incorporation of BrdUrd (Fig. 10*F*). The effects of PLP-J on its cellular targets were most impressive after 72 h of incubation (Figs. 9*C* and 10*C*). The minimal effective PLP-J concentration was 1 μg/ml (Figs. 9 (*B* and *C*) and 10 (*B* and *C*)). PLP-J also inhibited FGF2-stimulated endothelial cell proliferation (Fig. 10*G*). In summary, PLP-J stimulates uterine stromal cell proliferation and inhibits endothelial cell proliferation.

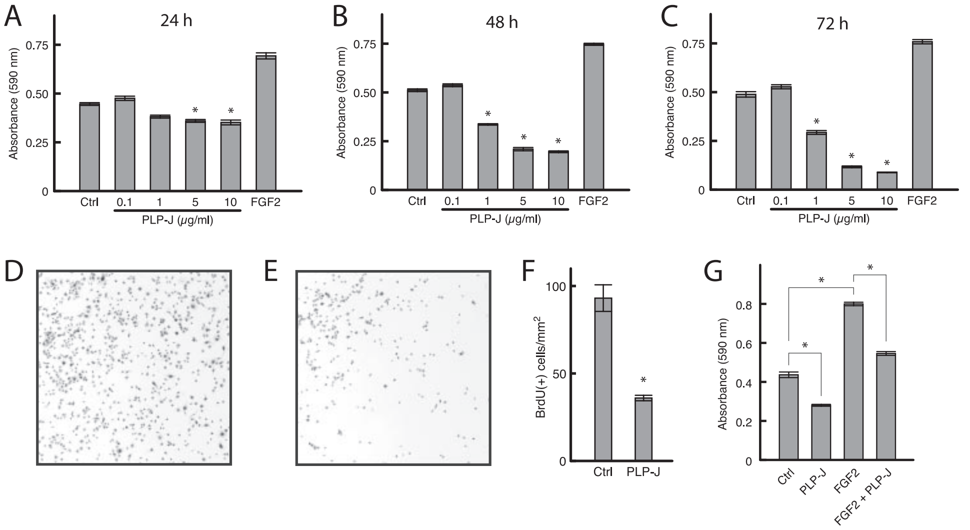

FIGURE 10. **PLP-J inhibits endothelial cell proliferation.** Endothelial cell proliferative responses to PLP-J were assessed by nonradioactive cell proliferation assay (*A–C*), crystal violet staining (*D* and *E*), and BrdUrd incorporation (*F*). Proliferative responses were evaluated at 24 h (*A*), 48 h (*B*), and 72 h (*C*) and expressed as absorbance at 590 nm. FGF2 (10 ng/ml) was used as a positive control. Values represent the mean ± S.E. of five replicates and are representative of three independent experiments. *D* and *E*, endothelial cells were treated with or without PLP-J (5 μg/ml) for 72 h and stained with 0.1% crystal violet. After washing, images were captured. *F*, effects of PLP-J on endothelial cell incorporation of BrdUrd. Cells were incubated in the presence of BrdUrd with or without PLP-J (5 μg/ml) for 16 h. BrdUrd-labeled cells were identified with a BrdUrd detection kit (BD Pharmingen). Results are expressed as the percentage of BrdUrd-stained cells *versus* the total number cells. *G*, endothelial cells were treated with vehicle (*Ctrl*), PLP-J (5 μg/ml), FGF2 (10 ng/ml), or FGF2 (10 ng/ml) plus PLP-J (5 μg/ml) for 72 h, and proliferation was assessed by a nonradioactive cell proliferation assay. All values represent the mean ± S.E. of five replicates. *Asterisks* indicate significant differences from controls ($p < 0.01$).

DISCUSSION

Evidence is accumulating for the involvement of the PRL family of cytokines in the regulation of maternal adaptations to pregnancy (11). In this report, we provide insights into a role for PLP-J, a member of the PRL family, in controlling intrauterine events during the establishment of pregnancy. The PLP-J protein was characterized, and its molecular and cellular interactions were investigated. PLP-J is expressed in uterine decidual cells and is a heparin-binding cytokine with potential paracrine actions on stromal and vascular cell development.

PLP-J is an intrauterine cytokine. The PLP-J protein is localized to a subpopulation of decidual cells situated proximal to the developing embryo (present study). This observation is consistent with earlier reports on PLP-J mRNA expression patterns (31–34). The decidual PLP-J protein has a molecular size of ~29 kDa and possesses heparin-binding properties. The expression profile and heparin binding features are also characteristics of another decidual PRL family cytokine, dPRP (22, 27, 29, 30, 39). PLP-J expression is also positively modulated by dPRP (16). This relationship may be indirect through dPRP promotion of decidual cell survival. In humans, PRL is the sole member of the PRL family (11, 62). It is expressed in both pituitary and extrapituitary sites, including decidual cells, and also possesses heparin binding properties (9, 10, 63). Rodent PRLs and most members of the PRL family do not exhibit heparin binding characteristics. The decidual cell expression of PLP-J, dPRP, and human PRL and their restricted distribution through interactions with heparin-containing molecules suggest that they act locally as cytokines within the vicinity of the embryo implantation site.

PLP-J interacts with and regulates specific cellular constituents of the uterus. Stromal cells and endothelial cells are among the putative intrauterine PLP-J targets. PLP-J can promote uterine stromal and endothelial cell adhesion. These interactions are mediated, at least in part, through heparin-containing molecules. Heparin-containing molecules are prominently displayed on the plasma membrane of cells and within the extracellular matrices of tissues (64). These molecules modulate development and are disrupted in some disease processes. PLP-J may not discriminate and instead associate with any uterine cell type possessing surface heparan sulfate proteoglycans in its proximity. Stromal cells and endothelial cells are prominent components of the preimplantation uterus, which undergo marked changes following embryo implantation (3, 5). Stromal cells serve as precursors for decidual cell differentiation, and endothelial cells line the intrauterine vasculature. During the establishment of pregnancy in mice and rats, the intrauterine vasculature undergoes specific regional modifications (3, 5, 65). Blood vessels in the mesometrial compartment (site of the developing chorioallantoic placenta) are significantly expanded in comparison with the anti-mesometrial uterine vasculature. In our *in vitro* analysis, PLP-J differentially affected the behavior of uterine stromal cells and endothelial cells. PLP-J stimulated proliferation of uterine stromal cells while inhibiting the proliferation of endothelial cells. These results indicate that PLP-J is a biologically active cytokine with the potential to regulate the intrauterine environment. The *in vivo* biological impact of decidual cell PLP-J production will be influenced by its access to its cellular targets and its relative contribution to the milieu of other growth factors and cytokines elaborated at the uteroplacental interface.

Relevant biologically active concentrations are difficult to determine for PLP-J. The heparin-binding properties of PLP-J will direct PLP-J to the cell surface or extracellular matrix, where it will have a juxtacrine mode of action. Thus, in an *in vivo* setting, PLP-J is sequestered and requires liberation from its tether to find its targets, or alternatively, the cellular targets may have to be brought to the sequestered PLP-J.

PLP-J modulates cell activities through mechanisms that are not yet defined. Some insights about PRL family member activation of signal transduction cascades are available. A subgroup of PRL family members (PRL, PL-I, and PL-II) utilizes the canonical PRL receptor signaling pathway (11). Among all members of the PRL family, PLP-J is most similar in amino acid sequence to the placental lactogens (31–34); however, PLP-J does not bind to or activate the PRL receptor (present study).

Apparently, the critical amino acids dictating PRL receptor recognition are not present in PLP-J. Other differences from its closest relatives (PL-I and PL-II) are also evident. PLP-J possesses a domain(s) facilitating interactions with heparin-containing molecules. This ligand-directed heparin binding may be a key to understanding the cellular actions of PLP-J. Some heparan sulfate proteoglycans, such as syndecans, are transmembrane proteins capable of interacting with heparin-binding growth factors and mediating the activation of signal transduction cascades (66, 67). Decidual cells, uterine stromal cells, and endothelial cells express members of the syndecan family, and PLP-J specifically binds to Sdc1. Thus, PLP-J could modulate cellular action directly via engaging transmembrane heparan sulfate proteoglycans (such as syndecans) or by interfering with the actions of other heparin-binding growth factors (*e.g.* fibroblast growth factors) that utilize transmembrane heparan sulfate proteoglycans. Differential expression of heparan sulfate proteoglycans could be responsible for the distinct effects of PLP-J on uterine stromal cells *versus* endothelial cells. Components of PLP-J activities were independent of interactions with heparin-containing molecules, suggesting that there may be additional modes of PLP-J action for some of its cellular targets. Among these may be interactions with specific membrane-associated receptor signaling pathways or possibly other glycosaminoglycans, including dermatan sulfate. PLP-J is capable of interacting with dermatan sulfate, and the uterine endometrium possesses dermatan sulfate proteoglycans (68). However, possible roles for dermatan sulfate proteoglycans in modulating PLP-J signaling have yet to be determined.

The PRL gene family expanded during the evolution of rodents (11, 17). New genes were derived from an ancestral template, which encode hormones and cytokines that are linked to viviparity and reproductive adaptations. PLP-J is part of the expanded rodent PRL family. It possesses a unique intrauterine tissue distribution and a novel intrauterine mode of action that is mediated, at least in part, through interactions with heparan sulfate proteoglycans.

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