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Placental Lactogen-I (PL-I) Target Tissues Identified with an Alkaline Phosphatase–PL-I Fusion Protein

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SUMMARY The rat placenta expresses a family of genes related to prolactin (PRL). Target tissues and physiological roles for many members of the PRL family have yet to be determined. In this investigation we evaluated the use of an alkaline phosphatase (AP) tag for monitoring the behavior of a prototypical member of the PRL family, placental lactogen-I (PL-I). A probe was generated consisting of a fusion protein of human placental AP and rat PL-I (AP–PL-I). The AP–PL-I construct was stably expressed in 293 human fetal kidney cells, as was the unmodified AP vector that served as a control. AP activity was monitored with a colorimetric assay in conditioned medium from transfected cells. Immunoreactivity and PRL-like biological activities of the AP–PL-I fusion protein were demonstrated by immunoblotting and the Nb2 lymphoma cell proliferation assay, respectively. AP–PL-I specifically bound to tissue sections known to express the PRL receptor, including the ovary, liver, and choroid plexus. Binding of AP–PL-I to tissues was specific and could be competed with ovine PRL. The results indicate that AP is an effective tag for monitoring the behavior of PL-I and suggest that this labeling system may also be useful for monitoring the actions of other members of the PRL family. (*J Histochem Cytochem* 46:737–743, 1998)

KEY WORDS

alkaline phosphatase fusion protein
ovary, corpus luteum prolactin receptors
liver, hepatic prolactin receptors
Nb2 lymphoma cells
placental lactogen-I
pregnancy
prolactin receptor

The prolactin (PRL) gene family contains at least 10 members some of which are expressed in the anterior pituitary, placenta, and/or uterus (Soares et al. 1998). Within this gene family, information is available on target cells and biological actions for only a subset of members utilizing the PRL receptor (classical members) and those regulating angiogenesis (Jackson et al. 1994; Volpert et al. 1996).

An important first step in understanding the actions of a hormone/cytokine is to identify its targets. Typically, target cells for various hormones/cytokines have been studied by radiolabeled ligand autoradiography or, when reagents are available to the designated ligand receptor, by immunocytochemical or *in situ* hybridization procedures. The former requires the isolation of a biologically active ligand to homogeneity and the lat-

ter requires identification and characterization of the receptor system used by the ligand. Neither of these options is readily available for nonclassical members of the PRL family whose biological actions during pregnancy are poorly understood. Flanagan and co-workers developed an alternative approach, involving the generation of alkaline phosphatase (AP)–ligand fusion proteins, that has proved particularly useful for identifying components of receptor tyrosine kinase signaling pathways, including ligands and receptors (Flanagan and Leder 1990; Cheng and Flanagan 1994; Cheng et al. 1995; Chiang and Flanagan 1995, 1996).

In this study, we have determined the effectiveness of utilizing an AP tag to monitor a prototypical member of the PRL family, placental lactogen-I (PL-I). PL-I is a glycoprotein, as are most members of the PRL family, and is secreted by trophoblast giant cells of the developing placenta from immediately postimplantation until midgestation (Faria et al. 1990). The actions of PL-I have been primarily studied via the generation of recombinant PL-I in heterologous cell types (Colosi et al. 1988; Robertson et al. 1994; Dai et al. 1996). PL-I has been shown to bind PRL receptors (MacLeod et al. 1989; Freemark et al. 1993) and possesses a

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number of actions previously attributed to pituitary PRL (Soares et al. 1998).

Collectively, the studies presented here indicate that the AP labeling system is an effective means of monitoring interactions of PL-I with its target cells and suggest the potential use of this labeling system for determining target cells for other PRL family members.

Materials and Methods

Reagents

Fetal bovine serum (FBS) and donor horse serum (HS) were purchased from Sigma (St Louis, MO). Reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad (Hercules, CA). All restriction enzymes, polymerases, and DNA ligase were purchased from New England Biolabs (Beverly, MA). The 293 cell line of human fetal kidney origin was obtained from American Type Culture Collection (Rockville, MD). Transformation competent Sure bacterial cells and random primer labeling kits were acquired from Stratagene (La Jolla, CA). DNA extraction kits were purchased from Qiagen (Chatsworth, CA). Nitrocellulose was obtained from Schleicher & Schuell (Keene, NH). Ovine PRL was purchased from Nobl Laboratories (Sioux City, IA). T7 DNA sequencing kits were acquired from United States Biochemical (Cleveland, OH). The pCMV/SEAP vector was acquired from Tropix (Bedford, MA). Radiolabeled nucleotides were purchased from DuPont-NEN (Boston, MA). Reagents for detection of immune complexes by enhanced chemiluminescence were acquired from Amersham (Arlington Heights, IL). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma.

Animals and Tissue Preparation

Holtzman rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were housed in an environmentally controlled facility, with lights on from 0600 to 2000 hr, and were allowed free access to food and water. Timed pregnancies and tissue dissections were performed as previously described (Soares et al. 1985; Faria et al. 1990). The presence of a copulatory plug or sperm in the vaginal smear was designated Day 0 of pregnancy. Protocols for the care and use of animals were approved by the University of Kansas Animal Care and Use Committee.

Generation of the AP-PL-I Fusion Protein

Construction of the AP-PL-I Vector. A fusion protein consisting of a modified human placental AP (PLAP) and rat PL-I was generated and used to monitor PL-I target cell interactions. PLAP, in its native form, is a heat-stable, membrane-associated AP. The carboxy terminus of PLAP mediates membrane binding. Using oligodeoxynucleotide-directed mutagenesis, Berger and co-workers (1988) engineered a carboxy-terminal truncation that resulted in a secreted PLAP referred to as SEAP. The coding sequence for SEAP was subsequently localized downstream of the CMV promoter in a vector containing ampicillin and neomycin resistance genes (pCMV/SEAP; Tropix). A nucleotide region representing the carboxy-terminal 197 amino acids of the mature rat PL-I protein was

amplified (Dai et al. 1996) and ligated into the pCMV/SEAP vector. Ligation with the PL-I insert resulted in a CMV promoter-driven vector containing the ligated cDNAs encoding a SEAP-PL-I fusion protein (AP-PL-I; see Figure 1). DNA sequencing of the insert was performed to verify the accuracy of the PCR amplification.

Transfection, Selection, and Cloning. After linearization with Bgl II, the AP-PL-I construct was electroporated into 293 cells. After a 2-week selection with 500 µg/ml G418, single clones were isolated by limiting dilution and screened for AP expression. An unmodified pCMV-SEAP vector (AP) was similarly transfected, and selected, and served as a negative control.

Preparation and Characterization of Medium Conditioned by the AP and AP-PL-I-transfected 293 Cells. Transfected 293 cells were cultured in MEM medium supplemented with 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS in an atmosphere of 5% CO₂/95% air at 37°C in a humidified incubator. After the cells reached confluence, the medium was changed to serum-free MEM + HEPES, further conditioned for 72 hr, collected and clarified by centrifugation, sterile-filtered (0.22 µm), and stored at -20°C until used. AP activity was measured from conditioned medium via a colorimetric assay. Initially, samples were heated for 30 min in a 65°C waterbath to inactivate endogenous heat-labile APs. Samples were then incubated at room temperature (RT) in a glycine buffer (50

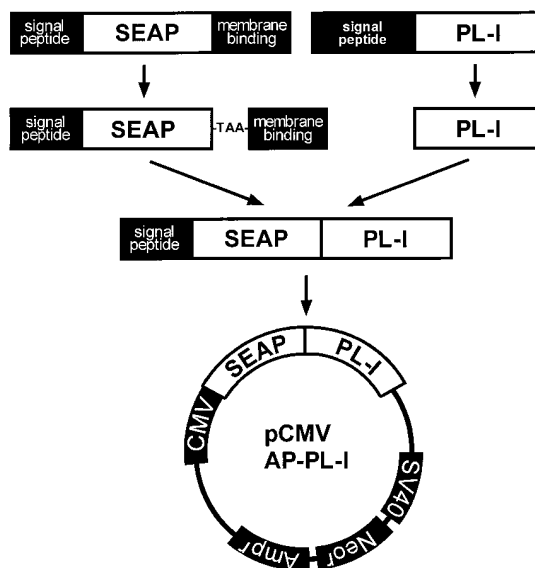


Figure 1 Construction of the AP-PL-I expression vector. A fusion protein consisting of a modified human placental AP (PLAP) and rat PL-I was generated and used to monitor PL-I target cell interactions. PLAP in its native form is a heat-stable, membrane-associated AP. The carboxy terminus of PLAP was truncated, resulting in a secreted PLAP referred to as SEAP and situated downstream of the CMV promoter in a vector containing ampicillin and neomycin resistance genes (pCMV/SEAP). A nucleotide region representing the carboxy-terminal 197 amino acids of the mature rat PL-I was amplified and ligated into the pCMV/SEAP vector. Ligation with the PL-I insert resulted in a CMV promoter-driven vector containing the ligated cDNAs encoding a SEAP-PL-I fusion protein (AP-PL-I).

mM glycine, pH 10.4, 0.5 mM MgCl₂, 0.5 mM ZnCl₂ containing nitrophenylphosphate (0.5 mg/ml) in a total reaction volume of 200 μ l. After a 5-min incubation absorbance was measured at 405 nm. One unit of AP is defined as the amount of enzyme that hydrolyzes 1 μ mole of *p*-nitrophenylphosphate to *p*-nitrophenol in 1 min at 37C in a volume of 1 ml.

Western Blot Analysis of PL-I

Western blot analysis was performed as previously described (Hamlin et al. 1994; Dai et al. 1996). AP-PL-I preparations were isolated from conditioned medium using immunoprecipitation with monoclonal antibodies to AP conjugated to agarose (Sigma). Samples were washed, eluted from agarose with treatment buffer, and separated by polyacrylamide gel electrophoresis in 7.5% gels under reducing conditions. Concentrated conditioned medium ($\times 10$) from differentiated Rcho-1 trophoblast cells served as a positive control for PL-I (Dai et al. 1996). Proteins from the gels were electrophoretically transferred to nitrocellulose. Polyclonal antibodies generated against a synthetic peptide corresponding to amino acids 1-19 of the mature PL-I protein (Hamlin et al. 1994) were used as probes. Immune complexes were detected using the enhanced chemiluminescence system as previously described (Dai et al. 1996).

Biological Characterization of AP-PL-I

PRL-like biological activities were assessed by use of the rat Nb2 lymphoma cell proliferation assay (Tanaka et al. 1980; Cohick et al. 1996). Nb2 lymphoma cells were routinely grown in Fischer's medium supplemented with 50 μ M 2-mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin, and containing both 10% HS and 10% FBS (maintenance medium = MM) in an atmosphere of 5% CO₂/95% air at 37C. Twenty-four hours before initiation of the assay, cells were harvested, washed with the supplemented 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 aliquotted into 16-mm wells (100,000 cells/ml/well) of a 24-well culture plate. Ovine PRL, AP, or AP-PL-I preparations were added at various concentrations and the cells incubated for an additional 72 hr. Samples of treated cells were collected and counted in a Sysmex Microcell counter (Model CC-110; TOA Medical Electronics, Tokyo, Japan). Treatments were performed in triplicate.

In Situ Analysis of AP-PL-I Binding to Rat Tissues

Tissues were frozen in liquid nitrogen and stored at -70C until tissue sections were prepared with the aid of a cryostat. Sections were mounted onto glass slides, washed in a modified Hank's balanced salt solution (HBHA; containing 20 mM HEPES, 0.5 mg/ml BSA, and 0.1% NaN₃), and incubated with AP, AP-PL-I, or AP-PL-I + excess PRL for 75 min at RT. After incubation, the sections were washed with HBHA supplemented with 0.1% Tween 20 and fixed for 2 min in a 20 mM HEPES buffer containing acetone (60%) and formaldehyde (3%). The fixed sections were washed, heated at 65C for 30 min to inactivate endogenous tissue AP activity, and then processed for detection of the heat-stable AP activity associated with the fusion proteins, and cover-

slips mounted in aqueous mounting medium. The AP and AP fusion protein were used at a concentration of 450 mU/ml. The specificity of binding was assessed by the addition of ovine PRL (5 μ g/ml) to some of the tissue section incubations.

Statistical Analysis

Data were analyzed by one-way ANOVA. The source of variation from significant F ratios was determined with Student's two-tailed *t*-test (Keppel 1973).

Results

As an important step towards demonstrating the suitability of the AP labeling system for monitoring activities of members of the PRL family, we generated and characterized an AP-PL-I fusion protein. The construction of the AP-PL-I vector was achieved by in-frame insertion of the cDNA sequence of mature rat PL-I downstream of the SEAP coding sequence within the pCMV-SEAP vector (Figure 1).

Generation and Characterization of the AP-PL-I Fusion Protein

The AP-PL-I construct and an unmodified AP control vector were transfected via electroporation into 293

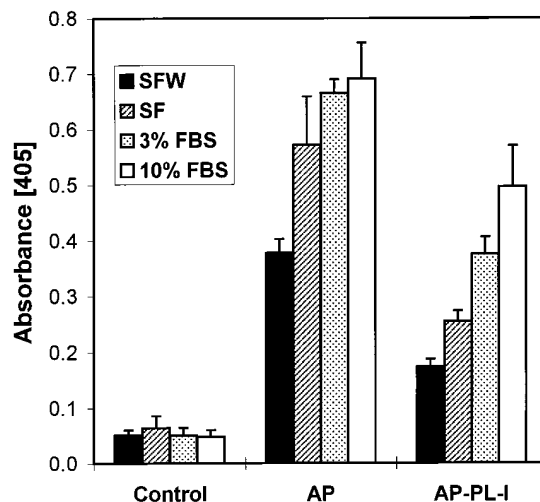


Figure 2 Alkaline phosphatase activity in conditioned medium from transfected and nontransfected cells. The AP-PL-I construct and an AP control construct were transfected via electroporation into 293 cells. After selection with G418, the highest expressing clones were identified and expanded. Transfected cells and non-transfected controls were seeded at 100,000 cells/ml into 24-well plates and cultured in MEM + HEPES + 10% FBS. After 24 hr, the cells were either washed with HBSS and the medium changed to serum-free MEM (SFW), serum-free MEM without washes (SF), MEM + 3% FBS (3% FBS), or to MEM + 10% FBS (10% FBS). After 72-hr incubation, conditioned medium was collected and heat-inactivated for 30 min in a 65C waterbath. Absorbance at 405 nm was measured after a 5-min incubation at room temperature of the sample with the nitrophenylphosphate substrate.

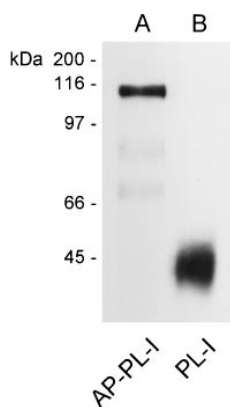


Figure 3 Western blot analysis of AP-PL-I. Samples were separated by polyacrylamide gel electrophoresis in 7.5% gels under reducing conditions and were electrophoretically transferred to nitrocellulose. Polyclonal antibodies generated against a synthetic peptide corresponding to amino acids 1–19 of the mature PL-I were used as probes. Immune complexes were detected using the enhanced chemiluminescence system. Lane A, AP-PL-I preparations were isolated from conditioned medium using immunoprecipitation with monoclonal antibodies to AP conjugated to agarose. Lane B, Concentrated conditioned medium ($\times 10$) from differentiated Rcho-1 trophoblast cells served as a positive control for PL-I. The AP-PL-I fusion protein migrated at an M_r approximating 110 kD, and trophoblast cell-produced PL-I migrated at M_r ranging from 36 to 45 kD.

cells. The presence of AP activity in conditioned medium from transfected cells and nontransfected cells was evaluated using a colorimetric assay. Several conditions for the production of recombinant protein were evaluated (Figure 2). Heat-stable AP activity secreted by AP- and AP-PL-I-transfected 293 cells was enhanced by the presence of FBS in the culture medium. The elevated AP activity in serum-containing cultures likely reflected stronger CMV promoter activity or possibly decreased protein degradation. Therefore, the generation of the AP-PL-I fusion protein did not interfere with AP enzymatic activity.

Anti-PL-I antibodies specifically recognized the AP-PL-I fusion protein. AP and the AP-PL-I fusion protein were initially enriched by immunoprecipitation with a monoclonal antibody to human PLAP conjugated to agarose. As shown by Western blot analysis, PL-I antibodies recognized an AP-PL-I protein species approximating 110 kD and native PL-I protein species synthesized by differentiated Rcho-1 trophoblast cells with sizes ranging from 36 to 45 kD (Figure 3). The

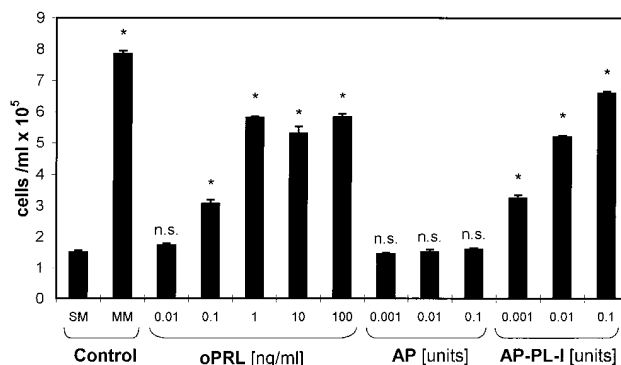


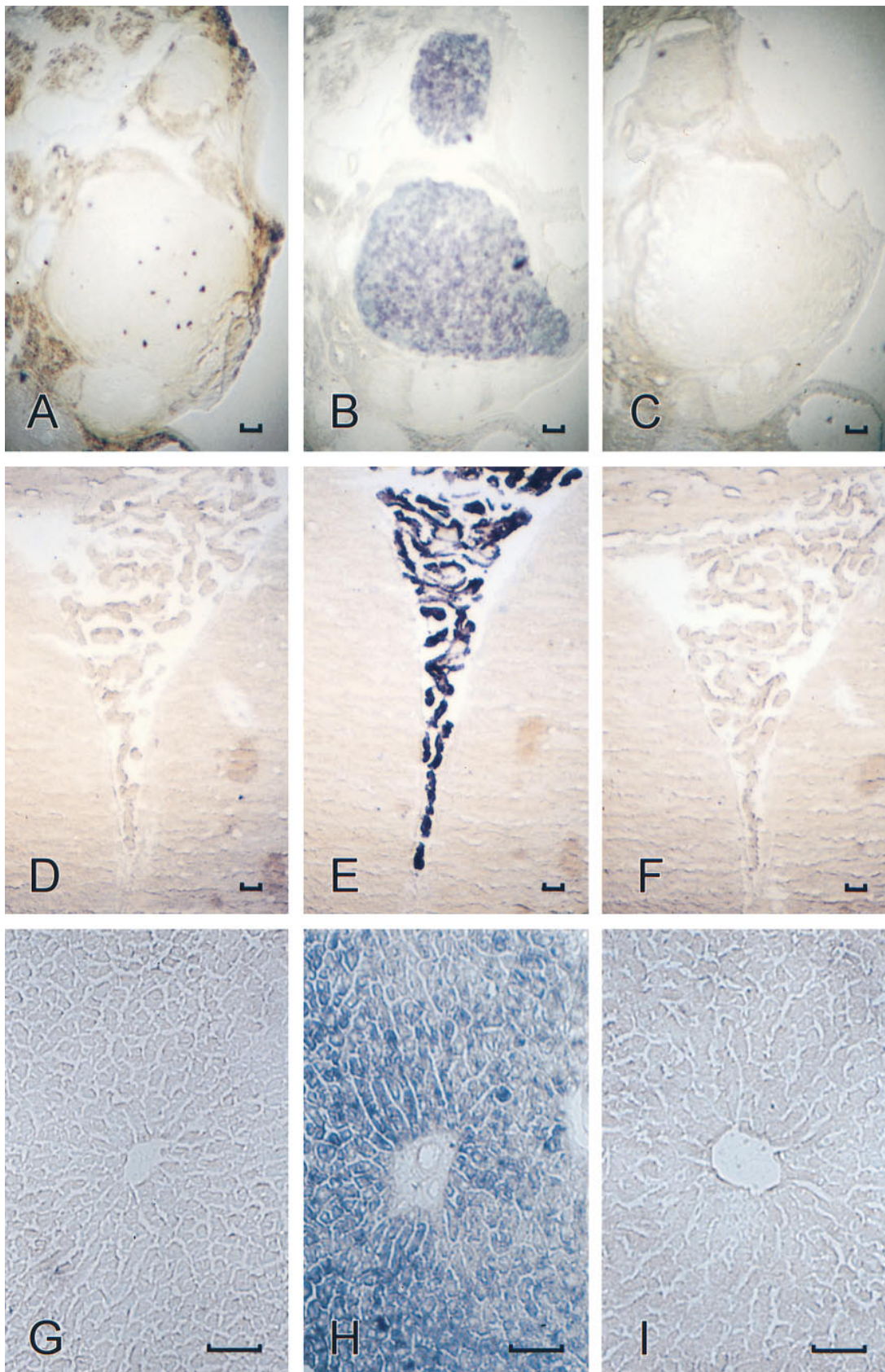
Figure 4 Effects of AP-PL-I on rat Nb2 lymphoma cell proliferation. PRL-like biological activities were assessed by use of the rat Nb2 lymphoma cell proliferation assay. Nb2 lymphoma cells were grown in supplemented Fischer's medium containing 10% HS and 10% FBS (maintenance medium = MM). Twenty-four hours before initiation of the assay, cells were harvested, washed with the supplemented 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 aliquotted into 16-mm wells (100,000 cells/ml/well) of a 24-well culture plate. Ovine PRL, AP, or AP-PL-I preparations were added at various concentrations and the cells incubated for an additional 72 hr and then counted. The AP and AP-PL-I preparations represent conditioned medium from 293 cells and are calibrated on the basis of AP activity. One unit of AP is defined as the amount of enzyme that hydrolyzes 1 μ mole of *p*-nitrophenyl-phosphate to *p*-nitrophenol in 1 min at 37C in a volume of 1 ml. Treatments were performed in triplicate. Results are presented as mean \pm SD. The PRL- and AP-PL-I-treated cultures significantly stimulated the proliferation of the Nb2 lymphoma cells compared to control cells in SM (* $p < 0.001$). n.s., not significant.

110-kD M_r of the AP-PL-I fusion protein was consistent with the predicted M_r of its AP and PL-I components. The AP control preparation was not recognized by the PL-I antibodies (data not shown).

Biological Characterization of AP-PL-I

For the AP-PL-I fusion protein to be a useful probe for monitoring PL-I interactions with its target tissues, the AP-PL-I should be able to mimic PRL biological actions. This issue was addressed by examining the actions of the AP-PL-I fusion protein on rat Nb2 lymphoma cells. The AP-PL-I fusion protein was capable of significantly stimulating the proliferation of rat Nb2 lymphoma cells in a concentration-dependent manner (Figure 4). Rat Nb2 lymphoma cell proliferation is dependent on activation of the PRL receptor signaling pathway, which can be achieved by PRL or

Figure 5 In situ analysis of AP-PL-I binding to rat tissues. Tissues sections were prepared with the aid of a cryostat. Sections were mounted on glass slides, washed, and incubated with AP (A,D,G), AP-PL-I (B,E,H), or AP-PL-I + PRL (C,F,I) for 75 min at room temperature. After incubation the sections were washed, fixed, washed, heated at 65C for 30 min to inactivate endogenous tissue AP activity, and processed for detection of the heat-stable AP activity. The specificity of binding was further assessed by the addition of ovine PRL (5 μ g/ml) to some of the tissue section incubations (C,F,I). (A–C) Ovary from Day 9 of pregnancy. (D–F) Frontal section of choroid plexus from female rat brain. (G–I) Liver from Day 9 of pregnancy. Bars = 100 μ m.



other ligands capable of interacting with the PRL receptor, such as PL-I (Tanaka et al. 1980; Colosi et al. 1988; Robertson et al. 1994; Cohick et al. 1996; Dai et al. 1996) (Figure 4). The AP control did not significantly stimulate rat Nb2 lymphoma cell proliferation (Figure 4).

Because the AP-PL-I fusion protein retained the ability to bind and activate PRL receptor signaling systems *in vitro*, we next evaluated its utility as an *in situ* probe for identifying PL-I target cells within tissue sections. AP, AP-PL-I, or AP-PL-I + excess ovine PRL were incubated with sections from several tissues, including ovaries from midgestation rats, liver tissues from midgestation rats, and brains from normal female rats (Figure 5). AP failed to demonstrate significant binding to any structures in the tissues investigated. AP-PL-I showed cell type-specific patterns of binding to the tissue sections that could be competed with excess ovine PRL (Figure 5). In the ovary, AP-PL-I was specifically localized to the corpus luteum, and binding within the brain was most prominent in the choroid plexus. AP-PL-I binding to liver sections was relatively homogeneous. On the basis of present evidence, the AP-PL-I fusion protein appears to be a useful probe for monitoring PL-I interactions with its target tissues and the localization of PRL receptors.

Discussion

In this report we have described the generation and characterization of an AP-PL-I fusion protein. The fusion protein was generated by stably expressing a fusion gene containing the heat-stable human placental AP cDNA upstream of the rat PL-I cDNA in the 293 human fetal kidney cell line. The AP-PL-I fusion protein retained AP enzymatic activity, PL-I immunological and biological activities, and represents an important advance in the generation of a probe for monitoring PL-I interactions with its target cells. Therefore, in addition to investigating growth factor receptor-tyrosine kinase interactions (Flanagan and Leder 1990; Cheng and Flanagan 1994; Cheng et al. 1995; Chiang and Flanagan 1995, 1996) and leptin-leptin receptor interactions (Tartaglia et al. 1995), the AP tag system also appears suitable for investigating the interactions of a class of ligands belonging to the PRL family with their receptors.

PL-I is a PRL receptor agonist expressed from implantation until midgestation by trophoblast giant cells situated at the maternal-placental interface (Colosi et al. 1988; MacLeod et al. 1989; Faria et al. 1990; Freemerk et al. 1993; Robertson et al. 1994; Dai et al. 1996; Soares et al. 1998). Recombinant PL-I has previously been shown to bind specifically to liver and ovarian membrane preparations (MacLeod et al. 1989), most likely via specific interactions with the extracel-

lular domain of the PRL receptor (Sakal et al. 1996). *In situ* analyses with radiolabeled recombinant PL-I have localized PL-I target cells to the decidua of the midgestation conceptus (Freemark et al. 1993) and to the hypothalamus and choroid plexus of the pregnant rat (Pihoker et al. 1993). We have similarly localized AP-PL-I binding to the maternal liver, choroid plexus, and corpus luteum of ovaries from pregnant rats. All of the *in vitro* and *in situ* analyses indicate that PL-I interacts with its target cells via PRL receptors. The interactions of PL-I with luteal cells is consistent with the actions of PL-I as a luteotropin (Galosy and Talamantes 1995) and the distribution of PRL receptor mRNAs in the corpus luteum during pregnancy (Clarke and Linzer 1993; Ouhtit et al. 1993). The complete distribution of PL-I tissue interactions in maternal, extraembryonic, and extraembryonic compartments during midgestation is yet to be resolved. In addition, it is not clear whether PL-I interacts with all PRL receptors or a subset of PRL receptors, and whether the activation of the PRL receptor signaling pathway in various target cells is identical to other activators of the PRL receptor system.

PL-I is a member of a larger family of hormones/cytokines that comprise the PRL family. PL-I is typical of most members of the PRL family in that it is a glycoprotein synthesized by trophoblast cells of the developing placenta (Soares et al. 1998). PL-I, PL-II, PL-I variant, and PRL are all capable of interacting with PRL receptors and activating the PRL receptor signaling pathway (Soares et al. 1998). Alternatively, there is a growing number of PRL family members that do not interact with PRL receptors (nonclassical members). These include PRL family members identified in the mouse: proliferin (Jackson et al. 1994), proliferin-related protein (Jackson et al. 1994), and likely a number of newly discovered PRL family members (H. Müller and M.J. Soares, unpublished results; D. Linzer, personal communication); the rat: PRL-like protein A (PLP-A) (Deb et al. 1993), PLP-B (Cohick et al. 1997), PLP-C (Conliffe et al. 1994; G. Dai, T. Takahashi, and M.J. Soares, unpublished results), PLP-C variant (G. Dai, T. Takahashi, and M.J. Soares, unpublished results), PLP-D (Iwatsuki et al. 1996), and decidual PRL-related protein (Rasmussen et al. 1996); and the cow: bovine PRL-related proteins I-VI (Schuler and Kessler 1992). Each of these ligands has unique structural properties and expression patterns within the uteroplacental compartment during pregnancy. Unfortunately, their biological actions are largely unknown. Classic approaches involving ligand purification, labeling, and characterization are tedious, time-consuming, and sometimes fraught with technical problems. On the basis of evidence provided in this report, the generation of AP-ligand fusion proteins may provide a relatively straightforward strategy for identifying bio-

logical targets for nonclassical members of the PRL family.

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