

Alkaline Phosphatase Fusion Proteins as Tags for Identifying Targets for Placental Ligands

Heiner Müller and Michael J. Soares

Summary

In this chapter, we describe protocols for the generation and characterization of alkaline phosphatase–ligand fusion proteins and their use as tools for the identification of specific ligand–receptor interactions.

Key Words: Alkaline phosphatase fusion protein; SEAP; protein tags; placenta; ligand–receptor interactions.

1. Introduction

Human placental alkaline phosphatase (PLAP) cDNA encodes 530 aa (17 aa signal peptide, 513 aa mature peptide). The C-terminus confers membrane binding. In an attempt to generate a new reporter gene with superior sensitivity and ease of use, Berger and coworkers (*1*) inserted a stop codon in front of the sequence that codes for membrane binding of the human PLAP. This translational terminator, which also generated an Hpa I site, truncated PLAP by 24 aa at the C-terminus. The resulting protein is secreted, consists of 506 aa (17 aa signal peptide, 489 aa mature peptide), and is referred to as the secreted form of human placental alkaline phosphatase (SEAP). Unlike the other AP isozymic forms (termed intestinal- and tissue-unspecific AP), PLAP and SEAP are unaffected by the presence of 10 mM homoarginine and extremely heat stable. The latter feature is very useful, because background AP activity can be virtually eliminated by simply heating the sample at 65°C for 30 min.

Typically, the tissue distribution of receptors for various ligands has been studied by radiolabeled ligand autoradiography or, when reagents are available, by immunocytochemical or *in situ* hybridization procedures. Flanagan and Brennan and their colleagues have utilized an alternative approach involving the generation of SEAP–ligand fusion proteins that has proven particularly

useful for identifying components of receptor tyrosine kinase signaling pathways, including ligands and receptors (2–9). Other groups utilized similar approaches, e.g., for the identification of the leptin receptor (10), a novel chemokine (11), and the monitoring of interactions between endothelial cells and leukocytes (12). The question arose whether this approach could be used to identify specific targets of placental proteins.

Rodent placentas prominently express an expanded family of genes that are structurally related to prolactin (PRL). The nomenclature for members of the PRL family reflects biological activities (placental lactogens [PLs]), structural relationships with PRL (PRL-like proteins [PLPs], PRL-related proteins [PRPs]), or associations with proliferation (proliferin). Those members of the PRL family that effectively mimic PRL action have been referred to as classical members of the PRL family. However, most members of the PRL family do not activate the PRL receptor and are referred to as nonclassical members. Before the application of the SEAP-tagging strategy, target tissues and physiological roles for most nonclassical members of the PRL family were unknown.

As a first step, the use of the AP tag for monitoring the behavior of a classical PRL family member was evaluated (13). Rat PL-I was chosen because (a) it is secreted by the developing placenta in a temporally and spatially specific pattern, (b) the recombinant protein was available, (c) targets with high expression of PRL receptors (PRL-R) were well known, (d) PL-I had been shown to bind the PRL-R, and (e) it possesses a number of actions previously attributed to pituitary PRL. Thus, the AP-PL-I model could be used to demonstrate that SEAP-tagging does not interfere with ligand binding and biological activities.

Using polymerase chain reaction (PCR)-cloning techniques, the cDNA for mature rat PL-I was inserted into the cytomegalovirus promoter (pCMV)-SEAP vector generating a probe that consisted of a fusion protein between human placental AP and rat PL-I (AP-PL-I). This construct was transfected into 293 cells. After a 2-wk selection with G418, single clones were isolated by limiting dilution and screened for AP expression. AP activity was monitored in the untreated, conditioned medium from transfected cells using a simple colorimetric assay. The unmodified AP vector was similarly transfected and selected and served as a control. Immunoreactivity was investigated by Western blotting. In vitro, AP-PL-I specifically bound to liver membranes, known to express high levels of PRL-R. The binding was specific because excess ovine PRL competed with AP-PL-I. PRL-like biological activities of the AP-PL-I fusion protein were successfully demonstrated using the rat Nb2 lymphoma cell proliferation assay. Binding of AP-PL-I to tissue sections was also specific and could compete with ovine PRL. Subsequently, the technique has been successfully applied to other members of the rodent PRL-gene family leading to the detection of their specific targets. The specific binding of PLP-A to natural

killer (NK) cells (**14**) and of decidual PRL-related protein (dPRP) to heparin and eosinophils (**15**) were identified with the help of SEAP-tagging. Other groups in the field adopted the technique and identified the binding of PLP-E and PLP-F to megakaryocytes (**16,17**) and of ovine PL to sheep uterine endometrial glands (**18**). Thus, the generation of SEAP–ligand fusion proteins provides a useful tool for the identification of specific ligand–receptor interactions.

2. Materials

1. The 293 human fetal kidney cells (American Type Culture Collection [ATCC], Manassas, VA or LGC Promochem, Teddington, UK).
2. 293 cell culture medium. Minimum essential medium (MEM) culture medium supplemented with 20 mM HEPES, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS).
3. pCMV-SEAP vector (Tropix, Inc., Bedford, MA).
4. Mouse monoclonal anti-human placental alkaline phosphatase (clone 8B6)-agarose (Sigma Chemical Co., St. Louis, MO, cat. no. A-2080).
5. Coplin jars (50 mL) (Fisher Scientific, Pittsburgh, PA).
6. HBS buffer: 20 mM HEPES, 150 mM NaCl, pH 7.0.
7. HBHA buffer: Hank's balanced salt solution with 0.5 mg/mL bovine serum albumin (BSA), 0.1% NaN₃, 20 mM HEPES, pH 7.0.
8. Acetone/formaldehyde fixative: 60% acetone, 3% formaldehyde in 20 mM HEPES, pH 7.0.
9. AP buffer: 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂.
10. AP stain: AP buffer containing 0.17 mg/mL 5-bromo,4-chloro,3-indolylphosphate (BCIP) and 0.33 mg/mL nitroblue tetrazolium (NBT).
11. Disposable vinyl specimen molds (e.g., 25 × 25 × 5 mm, Tissue-Tek, Ted Pella Inc., Redding, CA, cat. no. 4557).
12. Tissue freezing medium (Triangle Biomedical Sciences, Durham, NC or Fisher Scientific, cat. no. 15-183-13).
13. Coated slides (Fisher Scientific, e.g., Superfrost Plus, precleaned, cat. no. 12-550-15).
14. Hydrophobic marker (Pap Pen, Kiyota, Japan or Ted Pella Inc., cat. no. 22303).

3. Methods

We have used the AP-tagging strategy for investigating the biology of a number of members of the PRL family. The techniques described below are based on our experiences.

3.1. Generation of AP–Ligand Fusion Protein

1. A vector containing ampicillin and neomycin resistance genes and SEAP situated downstream of a CMV promoter (pCMV-SEAP) is commercially available.
2. A nucleotide region representing the mature ligand is then amplified using primers with Xba I sites (*see Note 1*) and ligated into the pCMV-SEAP vector linear-

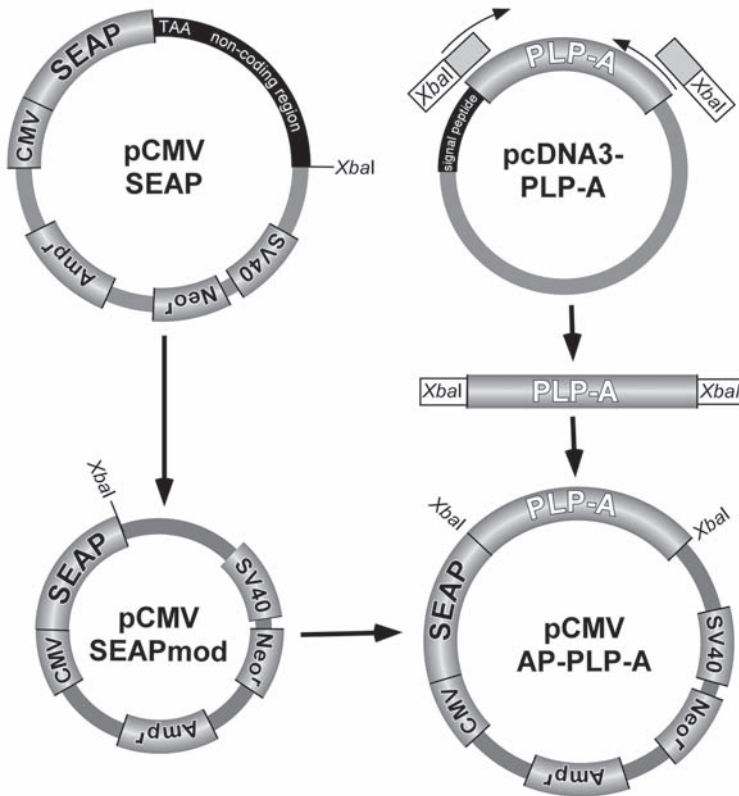


Fig. 1. Construction of the alkaline phosphatase (AP)-prolactin-like protein A (PLP-A) expression vector. A fusion protein consisting of a modified human placental AP and rat PLP-A was generated and used to monitor PLP-A target cell interactions. SEAP is situated downstream of the cytomegalovirus promoter (pCMV) in a vector containing ampicillin and neomycin resistance genes (pCMV-SEAP). From another vector, a nucleotide region representing the mature rat PLP-A was amplified and ligated into the modified pCMV-SEAP vector using the XbaI-site. Ligation with the PLP-A insert resulted in a CMV promoter-driven vector containing the ligated cDNAs encoding a secreted human placental AP (SEAP)-PLP-A fusion protein (AP-PLP-A).

ized at its unique Xba I site (**Fig. 1**). Ligation with the cDNA results in a CMV promoter driven vector containing the SEAP–ligand fusion gene. DNA sequencing of the insert is necessary to verify the orientation and the accuracy of the PCR amplification.

3. After linearization, the AP–ligand construct is electroporated into 293 human fetal kidney cells.
4. After a 2- to 3-wk selection with 500 mg/mL G418, single clones are isolated by limiting dilution and screened for AP expression.

5. An unmodified pCMV-SEAP vector (AP) should be similarly transfected, selected, and will serve as a negative control.
6. Transfected 293 cells are cultured in 293 cell culture medium in an atmosphere of 5% CO₂-95% air at 37°C in a humidified incubator.
7. After the cells reach confluence, the medium is changed to serum-free 293 cell culture medium, further conditioned for 72 h, collected and clarified by centrifugation, and filter sterilized (0.22 μm).
8. The achieved protein concentration can be determined by measuring AP-activity (see **Subheading 3.2.**). If needed, serum-free conditioned medium can be concentrated using membranes with a suitable molecular-weight cut-off. In our investigation, Amicon spiral cartridge concentrators were used at 4°C to prevent protein degradation. For small samples, spin columns with similar membranes work equally well. All conditioned media should be stored at -20°C until used.

3.2. AP Fusion Protein Characterization

AP activity can be measured from conditioned medium via a colorimetric assay. The AP tag can also be used to enrich the fusion protein.

3.2.1. AP Assay

1. Initially, samples are heated for 30 min in a 65°C water bath in order to inactivate endogenous heat labile AP.
2. Samples are then incubated at room temperature in a glycine buffer (50 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 μL.
3. Following a 5-min incubation, absorbance is measured at 405 nm.
4. One unit of AP activity is defined as the amount of enzyme that hydrolyzes 1 μmol of *p*-nitrophenylphosphate to *p*-nitrophenol in 1 min at 37°C in a volume of 1 mL.

3.2.2. AP-Ligand Isolation

AP-ligand preparations can be isolated from conditioned medium using immunoprecipitation or immunoaffinity chromatography with a monoclonal antibody to human PLAP (clone 8B6) conjugated to agarose (**14**).

3.2.3. Immunological Characterization

The AP-ligand fusion protein can be characterized by polyacrylamide gel electrophoresis and Western blotting with antibodies to human PLAP or with antibodies to the ligand (**13,14**).

3.2.4. Other Types of Biological Characterization

If in vitro bioassays are available for the ligand, then the AP fusion protein can be tested to determine whether the addition of the AP tag influences ligand biological activities. The Nb2 lymphoma cell proliferation assay represents a

highly sensitive *in vitro* assay for assessing classical PRL-like biological activities (**19**) (*see Note 2*).

3.3. Analysis of AP-PLP-A Binding to Tissues and Cells (see Note 3)

3.3.1. Tissue Preparation

1. A container is filled with enough liquid nitrogen to cover all samples. Sealable plastic bags and cryomolds are labeled. The cryomolds are filled with tissue freezing medium.
2. After the animal is sacrificed, target tissue is prepared as quickly as possible. The tissue is placed in the cryomold with the interesting side either up or down (sections will be transverse). Samples must be covered with tissue freezing medium.
3. With long forceps, cryomolds are placed onto liquid nitrogen and allowed to freeze from the bottom up while floating. Samples will sink when completely frozen.
4. When all the samples are frozen, they are taken out of the liquid nitrogen, wrapped in aluminum foil, put in sealable plastic bags, and placed in containers with dry-ice until finished. All samples are stored at -80°C until processed.
5. Alternatively, samples can be frozen directly in dry-ice-cooled heptane. Once frozen, the samples are transferred to suitable containers and stored at -80°C until processed.

3.3.2. Preparation of Frozen Sections

1. Tissue samples are equilibrated to temperature in the cryostat (e.g., -18 to -13°C).
2. Sections are cut 6–10 μm thick and, if necessary, flattened with a small brush.
3. Coated slides that will attract tissue sections electrostatically are used to pick up the tissue section by simply touching them.
4. Cytospins can be prepared to look for binding to cells in suspension.
5. All slides are frozen at -80°C until performance of the binding assay.

3.3.3. AP *In Situ* Binding Assay (see Fig. 2)

1. A 50-mL Coplin jar is filled with HBS buffer, covered with a lid, and placed in a 65°C water bath in preparation for the heat-inactivation step (*see Note 4*).
2. The AP or AP–ligand fusion protein solutions are prepared and stored on ice. Even for small sections, at least 200 μL is needed. To cover the entire surface of the slide, 1 mL is usually sufficient (*see Notes 5–7*).
3. Frozen sections or cytopins are taken out of the freezer. The white frost should dry out (this is not very time-critical, but allow to dry no longer than 1 h) (*see Note 8*).
4. Slides are labeled with a xylene-resistant marker or pencil and then soaked once for approx 5 min in HBHA Buffer.
5. Slides are drained and their backs, as well as their front edges, are wiped dry with gauze.

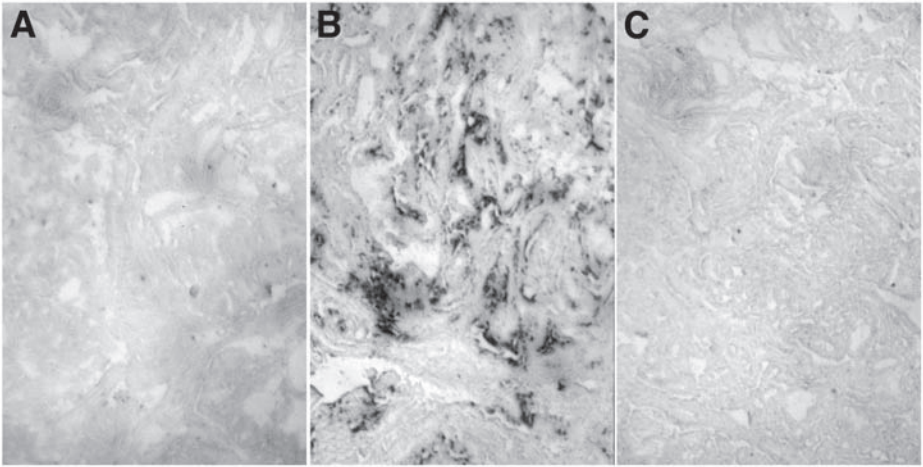


Fig. 2. *In situ* analysis of alkaline phosphatase (AP)-PL-I binding to tissue sections. Tissue sections were prepared with the aid of a cryostat. Sections were mounted on glass slides, washed, and incubated with AP (A), AP-PL-I (B), or AP-PL-I + prolactin (PRL) (C) for 75 min at room temperature. After incubation the sections were washed, fixed, washed, heated at 65°C 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 µg/mL) to the tissue section (C). (A–C) Mouse mammary tumor from pseudopregnant mice of the DDD strain of *Mus musculus* carrying the mouse mammary tumor virus (MMTV), 40× magnification.

6. Samples are overlaid with fusion protein supernatant for 75 min at room temperature in a humidified chamber (moist and level). Sections should not be allowed to become dry.
7. Samples are washed six times for 5 min in HBHA buffer + 0.1% Tween 20. The slides are drained by tapping on paper towels, rinsed briefly in the first wash, and placed in the second wash. They are agitated at the beginning, middle, and end of each wash.
8. Tissue sections are fixed with acetone/formaldehyde fixative for 2 min.
9. Slides are washed three times for 5 min with HBS buffer.
10. To heat-inactivate endogenous AP, slides are transferred into a Coplin jar filled with HBS buffer and then to a water bath already adjusted to 65°C.
11. After 30 min, slides are transferred to another Coplin jar containing AP buffer to be cooled and rinsed in order to pre-adjust pH and temperature.
12. Slides are drained and their backs and front edges wiped dry with gauze. Sections should not be allowed to become dry.
13. Sections are overlaid with AP Stain for 2 h in a humidified chamber.

14. The process of staining is monitored on a white background (e.g., wet white filter paper). The required staining time can vary between 10 min and 16 h. When staining is sufficient, stain is drained off onto a paper towel (*see Note 9*).
15. Slides are rinsed five times in distilled water and then air-dried for 30 min. Ethanol/xylene dehydration should be avoided because the precipitated stained material will wash out.
16. Immediately before coverslipping, slides are rinsed briefly (<1 min) in xylene.
17. Following coverslipping with Permount (aqueous mounting medium), the slides are air-dried (usually approx 3 d at room temperature).

4. Notes

1. The cDNA insert (corresponding to the protein of interest) must be subcloned in-frame with the SEAP-coding sequence and without its signal peptide. Glycine may serve as a linker.
2. AP-tagged PL-I retains its PRL-like biological activities (**13**). This need not be the case for all AP-tagged ligands. In some cases, the AP tag may disrupt and/or modify ligand–receptor interactions.
3. Others have successfully adopted the SEAP-tagging strategy for the identification of targets for placental ligands (**17,18**). There are also examples of using the AP–ligand fusion proteins as a screening tool in expression cloning strategies for identification of the ligand’s receptor (**10**).
4. The exact temperature is critical for effective heat-inactivation of background AP activity. Therefore, the waterbath should adjust to 65°C for at least 1 h before using.
5. Two different negative controls should be used: the serum-free cell culture medium for 293 cells to check for successful heat inactivation of endogenous AP in the tissue, and SEAP containing conditioned medium, to check for binding related to SEAP itself. The specificity of binding can be assessed by incubation with the AP control or addition of various peptide hormones.
6. To achieve good staining, 100–500 mU/mL of fusion protein are needed. To calculate the amounts of tagged protein, the following estimation could be useful: SEAP: 1000 mU = 1 µg, SEAP-PRL family ligand: 600 mU approx 1 µg. The enzymatic activity per mass of SEAP fusion protein depends on the molecular weight ratio between SEAP and the tagged protein.
7. The application of the technique to another nonclassical member of the PRL family, dPRP, turned out to be more difficult as a result of the heparin-binding of dPRP. Wang et al. (**15**) demonstrated that AP–dPRP fusion protein bound readily to heparan sulfate-containing wild-type Chinese hamster ovary (CHO) cells but not to heparan sulfate-deficient CHO-pgsD-677 cells. Using heparan sulfate-deficient CHO cells transiently transfected with the long form of the rat PRL-R, it could be shown that AP–dPRP failed to bind the PRL-R. In contrast, AP–PL-I, a known ligand of the PRL receptor, effectively bound to these PRL receptor-transfected cells. Binding assays showed that AP–dPRP bound to virtually all components of the uterus. The challenge was to overcome the strong affinity to heparin

and to show heparin-independent binding. By pretreatment with heparitinase and/or heparin, dPRP binding to tissues was dramatically affected. A population of non-heparin binding sites was identified by consecutively incubating tissue sections with AP-d/tPRP followed by excess heparin (250 $\mu\text{g}/\text{mL}$ to 10 mg/mL). This strategy finally led to the identification of discrete d/tPRP target cells within the endometrium and myometrium of the nonpregnant rat.

8. Circling the tissue sections with a hydrophobic marker helps to save reagents.
9. Both reagents used in the AP histochemical assay, NBT and BCIP, are toxic (especially the dust). Wearing gloves and careful handling is mandatory. A stock solution should be prepared and stored at 4°C. Tubes should be covered with aluminum foil because both AP substrates are light-sensitive.

Acknowledgments

The authors would like to thank Belinda M. Chapman, Christopher B. Cohick, Bing Liu, and Guoli Dai for their participation in the development of the procedures and techniques. This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) of Germany (Mu 1183 1-1, Mu 1183 3-1), the National Institutes of Health (NIH) (HD20676, HD39878), and the Hall Family Foundation.

References

1. Berger, J., Hauber, J., Hauber, R., Geiger, R., and Cullen, B. R. (1988) Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* **66**, 1–10.
2. Brennan, C., Monschau, B., Lindberg, R., et al. (1997) Two Eph receptor tyrosine kinase ligands control axon growth and may be involved in the creation of the retinotectal map in the zebrafish. *Development* **124**, 655–664.
3. Brennan, C. and Fabes, J. (2003) Alkaline phosphatase fusion proteins as affinity probes for protein localization studies. *Sci STKE* **2003(168)**, PL2.
4. Cheng, H. J. and Flanagan, J. G. (1994) Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. *Cell* **79**, 157–168.
5. Cheng, H. J., Nakamoto, M., Bergemann, A. D., and Flanagan, J. G. (1995) Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell* **82**, 371–381.
6. Chiang, M. K. and Flanagan, J. G. (1995) Interactions between the Flk-1 receptor, vascular endothelial growth factor, and cell surface proteoglycan identified with a soluble receptor reagent. *Growth Factors* **12**, 1–10.
7. Chiang, M. K. and Flanagan, J. G. (1996) PTP NP, a new member of the receptor protein tyrosine phosphatase family, implicated in development of nervous system and pancreatic endocrine cells. *Development* **122**, 2239–32250.
8. Flanagan, J. G. and Leder, P. (1990) The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell* **63**, 185–194.

9. Flanagan, J. G. and Cheng, H. J. (2000) Alkaline phosphatase fusion proteins for molecular characterization and cloning of receptors and their ligands. *Methods Enzymol.* **327**, 198–210.
10. Tartaglia, L. A., Dembski, M., Weng, X., et al. (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83**, 1263–1271.
11. Nagira, M., Imai, T., Hieshima, K., et al. (1997) Molecular cloning of a novel human CC chemokine secondary lymphoid-tissue chemokine that is a potent chemoattractant for lymphocytes and mapped to chromosome 9p13. *J. Biol. Chem.* **272**, 19,518–19,524.
12. Fong, A. M., Erickson, H. P., Zachariah, J. P., et al. (2000) Ultrastructure and function of the fractalkine mucin domain in CX(3)C chemokine domain presentation. *J. Biol. Chem.* **275**, 3781–3786.
13. Müller, H., Dai, G., and Soares, M. J. (1998) Placental lactogen-I (PL-I) target tissues identified with an alkaline phosphatase-PL-I fusion protein. *J. Histochem. Cytochem.* **46**, 737–743.
14. Müller, H., Liu, B., Croy, B. A., et al. (1999) Uterine natural killer cells are targets for a trophoblast cell-specific cytokine, prolactin-like protein-A. *Endocrinology* **140**, 2711–2720.
15. Wang, D., Ishimura, R., Walia, D. S., et al. (2000) Eosinophils are cellular targets of the novel uteroplacental heparin-binding cytokine decidual/trophoblast prolactin-related protein. *J. Endocrinol.* **167**, 15–28.
16. Lin, J. and Linzer, D. I. (1999) Induction of megakaryocyte differentiation by a novel pregnancy-specific hormone. *J. Biol. Chem.* **274**, 21,485–21,489.
17. Zhou, B., Lum, H. E., Lin, J., and Linzer, D. I. (2002) Two placental hormones are agonists in stimulating megakaryocyte growth and differentiation. *Endocrinology* **143**, 4281–4286.
18. Noel, S., Herman, A., Johnson, G. A., et al. (2003) Ovine placental lactogen specifically binds to endometrial glands of the ovine uterus. *Biol. Reprod.* **68**, 772–780.
19. Tanaka, T., Shiu, R. P. C., Gout, P. W., Beer, C. T., Noble, R. L., and Friesen, H. G. (1980) A new sensitive and specific bioassay for lactogenic hormones: measurement of prolactin and growth hormone in human serum. *J. Clin. Endocrinol. Metab.* **51**, 1058–1063.