Heterologous Expression and Characterization of Prolactin-like Protein-A

IDENTIFICATION OF SERUM BINDING PROTEINS*

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In this report, we describe the heterologous expression of prolactin-like protein-A (PLP-A) in Chinese hamster ovary (CHO) cells, the characterization of recombinant PLP-A, and the identification of serum PLP-A-binding proteins. CHO cell and native placental PLP-A showed similar immunoreactive characteristics and electrophoretic mobilities. N-terminal sequencing verified the identity and purity of the recombinant PLP-A species and the site of cleavage of the signal peptide from the mature secreted PLP-A species. Recombinant PLP-A lacked activity in standardized prolactin and growth hormone in vitro bioassays. Antibodies generated to recombinant PLP-A facilitated the cellular localization of PLP-A and the identification of high molecular weight PLP-A complexes. Cross-linking analyses of radioiodinated PLP-A with serum harvested from late gestation rats indicated the presence of two major cross-linked complexes migrating under reducing conditions at 130 and 250 kDa and two minor cross-linked complexes migrating at 70 and 110 kDa. Binding of PLP-A to serum proteins was specific for PLP-A and not effectively competed by other members of the prolactin/growth hormone family. The PLP-A binding species were also found in serum from non-pregnant female and male rats.

Prolactin-like protein-A (PLP-A)1 is one of at least six different structurally related proteins abundantly expressed by the rat placenta (Soares et al., 1991). PLP-A is a member of the prolactin (PRL)/growth hormone (GH) family. Genes for PLP-A, PRL, and relatives expressed in the placenta are localized to chromosome 17 of the rat genome whereas the GH gene is present on chromosome 10 (Cooke et al., 1986; Levan et al., 1991; Deb et al., 1991c). Unlike the pituitary-specific rat PRL protein, PLP-A is a glycoprotein and secreted in large amounts by trophoblast cells of the choioallantoic placenta during the second half of gestation (Campbell et al., 1989; Deb et al., 1989b). PLP-A is synthesized as a 25 kDa precursor that is glycosylated to 29 and 33 kDa species (Deb et al., 1989b). In circulation, PLP-A exists as a high molecular weight complex (Deb and Soares, 1990) reminiscent of the behavior of GH in blood (Baumann, 1991). Although, the biological actions of PLP-A are presently unknown, the structural relationship of PLP-A to PRL, GH, and other members of the cytokine superfamiliy (Bazan, 1990; Soares et al., 1991) suggests a possible role for PLP-A in coordinating metabolic adjustments between fetal and maternal compartments (see Soares et al., 1991, for review). As a first step in attempting to understand the role of PLP-A in pregnancy, we have generated recombinant PLP-A, investigated the activities of PLP-A in PRL and GH in vitro biological assays, and examined the nature of circulating high molecular weight PLP-A complexes.

MATERIALS AND METHODS

Reagents

Reagents for polyacrylamide gel electrophoresis and Affi-Gel Blue affinity chromatography resin were purchased from Bio-Rad. Adjuvant for immunizations was obtained from Organon Teknika Corp. (West Chester, PA). Methotrexate was purchased from Calbiochem (San Diego, CA). All restriction enzymes, polymerases, and DNA ligase were purchased from New England Biolabs (Beverly, MA). Nitrocellulose was obtained from Schleicher and Schuell. Human placental lactogen (PL) and rat and ovine PRL were obtained from the National Hormone and Pituitary Program (Baltimore, MD). Bovine GH was obtained from United States Department of Agriculture (Beltsville, MD). Antibodies to mouse α2-macroglobulin were obtained from Nordic Immunological Laboratories (Capiatano Beach, CA). The cross-linking reagent, bi(sulfosuccinimidyl) suberate, and the iodination reagent, Iodo-Gen, were obtained from Pierce Chemical Co. Streptavidin-biotin immunoperoxidase kits for rabbit immunoglobulin G were obtained from Zymed Laboratories (South San Francisco, CA). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma.

Animals and Tissue Preparation

Holstein rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were housed in an environmentally controlled facility, with lights on from 0600-2000 h, and allowed free access to food and water. Timed pregnancies and tissue dissections were performed as previously described (Soares, 1987). The presence of a copulatory plug or sperm in the vaginal smear was designated day 0 of pregnancy. Blood was collected by decapitation from pregnant rats (at different days of pregnancy), non-pregnant female rats, and male rats. Blood was centrifuged at 3000 revolutions/minute for 15 min, and serum was collected. Placental cytosol was prepared as previously described (Deb et al., 1989a). Protein concentrations of

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sample preparations were estimated by the method of Bradford (1976). Protocols for the care and use of animals were approved by the University of Kansas Animal Care and Use Committee.

Generation of Recombinant PLP-A

Construction of the PLP-A Expression Vector—The PLP-A cDNA used in these experiments was obtained as a gift from Drs. Mary Lynn Duckworth and Henry G. Friesen (University of Manitoba, Manitoba, Canada; Duckworth et al., 1980). The expression vector, pMSXND, was originally constructed by Drs. S.-J. Lee and Daniel Nathans of Johns Hopkins University for the generation of recombinant mouse prolactin (a member of the mouse placenten PRL family; Lee and Nathans, 1988). This expression vector contains a metallothionin promoter, SV40 splicing, and polyadenylation signals, a neomycin resistance gene, and a dihydrofolate reductase gene (Fig. 1). The protocol we have used is based largely on that previously described by Linzer and colleagues (Colosi et al., 1988a, 1988b).

A 625-base pair EcoRI fragment containing the entire coding sequence of PLP-A was isolated from plasmid pRP6-5 after electrophoretic separation in a 5% polyacrylamide gel. Termini of the isolated fragment were filled with Klenow fragment of DNA polymerase I. XhoI linkers were added to the ends, enabling the PLP-A cDNA to be inserted into the unique XhoI site of the pMSXND vector. Subcloning at the XhoI site situates the PLP-A insert downstream of the mouse metallothionin promoter and upstream of the SV40 splicing and polyadenylation signals. Recombinant plasmid DNA was purified from *Escherichia coli* JM105 cells by lysozyme and alkaline-SDS lysis of bacterial cultures, followed by two bandings on CsCl-ethidium bromide density gradients (Sambrook et al., 1989). The orientation of the PLP-A cDNA in the expression vector was confirmed by HindIII and BamHI digestion. The plasmid containing the correct PLP-A insert orientation was designated as pRPLPA.

Transfection, Selection, and Amplification—Chinese hamster ovary cells (CHO) obtained from the American Type Culture Collection (ATCC CCL-61, CHO-K1, Rockville, MD) were used as the host for the pRPLPA expression vector. CHO cells were routinely maintained in DMEM/MCDB-302 culture medium containing 1 mM proline, antibiotics, and 10% fetal bovine serum. Transfection was carried out by electroporation according to the procedure described by Bothwell et al. (1990). Following electroporation, the cells were transferred to tissue culture flasks containing DMEM/MCDB-302 culture medium containing the supplements described above. Forty-eight h following electroporation, the selection process was initiated.

Selection was performed with the neomycin analog, Geneticin (G418). The cells were transfected to minimal essential medium containing proline, antibiotics, and G418 at a final concentration of 1 mg/ml. Once sizable colonies of cells were evident the amplification process was initiated.

Amplification takes advantage of the sensitivity of the dihydrofolate reductase construct present in the pMSXND vector to methotrexate. CHO-transfected and selected cells were exposed to successive increases in methotrexate (in the G418 selection medium). The methotrexate concentrations were increased every 2–3 weeks in a step wise manner (starting with 0.1 μM and concluding with 200 μM).

Expression of recombinant PLP-A protein was monitored by Western blot analysis.

**Generation of Conditioned Medium from CHO Cells Transfected with the PLP-A Expression Vector**—After selection and amplification the transfected CHO cells were grown to confluence in DMEM/MCDB-302 medium containing 1% fetal bovine serum. The monolayers were washed with DMEM/MCDB-302 medium without serum and cultured in the same serum-free medium supplemented with 100 nm cadmium chloride. The medium conditioned by the monolayers were collected and replaced with fresh medium at 24-h intervals for at least 12 days. The first 24-h collection was discarded because of the presence of residual serum. The remainder of the conditioned medium was pooled and stored frozen at −80 °C until further analysis.

**Purification of Recombinant PLP-A**—Approximately 8 liters of conditioned medium were processed in three different batches. Conditioned medium was thawed and centrifuged at 19,000 × g for 20 min to remove debris. Zinc sulfate was added to the supernatant to a final concentration of 100 mM and stirred for 30 min. The resulting precipitates were collected by centrifugation at 10,000 × g for 15 min. Pellets were then dissolved in 500 mM Na-EDTA, pH 7.5, and dialyzed against two changes of distilled water (6 liters each) and finally phosphate-buffered saline (PBS: 10 mM sodium phosphate, 150 mM NaCl, pH 7.4). The dialysate was then subjected to concanavalin A affinity chromatography as previously described (Deb et al., 1989b). Proteins specifically eluted from the concanavalin A-Sepharose column with α-methyl mannoside (0.2 M) and N-acetylglucosamine (0.2 M) were concentrated by ultrafiltration and chromatographed on a Sephacryl S-200 column (3 × 92 cm), equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 300 mM NaCl. Amino acid analysis of the flow rate was maintained at 20 ml/h. Fractions collected from the gel filtration column were monitored for absorbance at 280 nm and for PLP-A immunoreactivity by enzyme-linked immunoassay or by Western blot analysis (Deb et al., 1989a, 1989b).

All steps in the purification were performed at 4 °C. Separation of the 29- and 35-kDa PLP-A species was achieved by SDS-polyacrylamide gel electrophoresis and electroelution (Deb and Soares, 1990).

**Characterization of Recombinant PLP-A**

**Amino-terminal Sequence Analysis**—Amino acid sequences were determined by the Edman degradation procedure, using a gas-phase protein sequencer as previously described (Deb et al., 1991a, 1991b). Approximately 20 μg of the 29- and 35-kDa PLP-A protein species were sequenced.

**Generation of Antibodies to Recombinant PLP-A**—Purified recombinant 33-kDa PLP-A (approximately 100 μg) was used to immunize a New Zealand White rabbit (Camm Research Laboratories, Wayne, NJ). Preimmune serum, N-acetylglucosamine (0.2 M) were concentrated by ultrafiltration and chromatographed on a Sephacryl S-200 column (3 × 92 cm), equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 300 mM NaCl. Amino acid analysis of the flow rate was maintained at 20 ml/h. Fractions collected from the gel filtration column were monitored for absorbance at 280 nm and for PLP-A immunoreactivity by enzyme-linked immunoassay or by Western blot analysis (Deb et al., 1989a, 1989b). Samples were separated by SDS-polyacrylamide gel electrophoresis in 7.5 or 10% gels under reducing or non-reducing conditions. Proteins were electrophoretically transferred to nitrocellulose. Antibodies generated to synthetic peptides corresponding to three different regions of the PLP-A sequence (amino acids 101–114, 129–145, and 152–164; Deb et al., 1989b) and to recombinant PLP-A were used in the Western blot analyses. In some experiments, preimmune serum, antibodies saturated with the respective purified antigen, or antibodies directed against amino acids 56–70 of rat PLP-II (Deb et al., 1989a) were used as controls.

**PRL and GH in Vitro Bioassays**—PRL-like and GH-like bioactivities of PLP-A were assessed by evaluating the effects of purified recombinant PLP-A on the proliferation of Nb2 rat lymphoma cells and on the differentiation of mouse 3T3-F442A cells, respectively. PRLs stimulate proliferation of Nb2 rat lymphoma cells (Gout et al., 1980), and GHs stimulate adipocyte differentiation of 3T3-F442A cells (Morikawa et al., 1982).

The Nb2 cell proliferation assay was conducted as described previously (Tanaka et al., 1980) with minor modifications (Deb et al., 1991a). The adipocyte conversion 3T3-F442A assay was performed according to the procedure of Green and co-workers (Morikawa et al., 1984; Djan et al., 1985). Briefly, exponentially growing 3T3-F442A cells were plated at a density of 35 000 cells/cm². After 3–4 days (near confluence), growth medium (DMEM with 5% cat serum and 0.5% calf serum) was replaced with conversion medium.

![Diagram of the PLP-A expression vector](https://example.com/diagram)

**Fig. 1. Schematic representation of the PLP-A expression vector, pRPLPA.** The filled box represents the location of the PLP-A cDNA inserts. Abbreviations: SV40, splicing and polyadenylation signals of SV40; mdhfr, mouse dihydrofolate reductase gene; G418', neomycin resistance gene; Amp', ampicillin resistance gene; Mt1, mouse metallothionein I promoter.
Electrophoretically separated in the absence (lanes A and B) or by CHO cells (lanes C and D) with antipeptide antibodies to PLP-A. Placental cytosolic preparations and conditioned medium generated from CHO cells transfected with the pRPLP-A expression vector were electrophoretically separated in 12.5% gels, transferred to nitrocellulose, and examined by Western blot analysis. Lanes A and C were incubated with antipeptide antibodies to amino acids 101-114 of the PLP-A sequence. Lanes B and D were incubated with same antipeptide antibodies saturated with antigen. M, standards (×10^{-3}) are shown. Middle panel, examination of the reactivity of PLP-A produced by CHO cells with antibodies directed to various regions of the PLP-A sequence (lanes A-C) and to PL-II (lane D). Proteins were electrophoretically separated in 12.5% gels, transferred to nitrocellulose, and examined by Western blot analysis. Antisera directed to amino acids 101-114 (lane A), 129-145 (lane B), and 152-164 (lane C) of the PLP-A sequence, and amino acids 56-70 of the PL-II sequence (lane D) were each used at a final dilution of 1:500. M, standards (×10^{-3}) are shown. Bottom panel, stability of the responsiveness of the engineered CHO cells to cadmium chloride. Proteins isolated from medium conditioned by engineered CHO cells after 3 (lanes A and B) or 10 (lanes C and D) days of serum-free culture in the absence (lanes A and C) or presence (lanes B and D) of 100 nM cadmium chloride were examined by Western blot analysis using antibodies directed to amino acids 152-164 of the PLP-A sequence. Specificity of the immunoreactions was determined by comparing the reactivity of the antibodies with antibodies absorbed with excess antigen.

Identification of PLP-A Serum-binding Proteins

Evidence for the existence of PLP-A serum-binding proteins was derived from Western blotting experiments using antibodies to recombinant PLP-A and from experiments using radiolabeled recombinant PLP-A and the cross-linking reagent bis(sulfosuccinimidy1) suberate.

Cross-linking Experiments with Radiolabeled Recombinant PLP-A—The identity of complexes formed between radiolabeled PLP-A and serum proteins was determined following cross-linking, electrophoresis, and autoradiography. Radiolabeling was carried out using the iodination reagent Iodo-Gen as previously described (Soares et al., 1988). The radiolabeled protein (2 × 10^6 cpm) was incubated overnight with diluted rat serum (100 μl, 1:1 ratio of serum to PBS) at 4°C in the absence or presence of excess recombinant PLP-A, ovine PRL (100 μg), bovine GH (100 μg), or human PL (100 μg each). Cross-linking reactions were carried out at room temperature for 30 min in the presence of 1 mM bis(sulfosuccinimidy1) suberate solution in sodium phosphate buffer (50 mM, pH 7.4). The reaction was stopped by addition of the same sodium phosphate buffer containing 50 mM ethanalamine and 20 mM N-ethylmaleimide. Cross-linked proteins were concentrated by acetone precipitation or concentrated and further purified by immuno precipitation with antibodies (Deb et al., 1989b). Samples were then separated by SDS-polyacrylamide gel electrophoresis under reducing conditions, the gels dried, and the migration of radioactive proteins determined by autoradiography.
RESULTS

Heterologous Expression of PLP-A—Medium conditioned by CHO cells transfected with the PLP-A expression vector (pRPLPA) contained proteins that possessed immunochemical and electrophoretic characteristics similar to native placental PLP-A (Fig. 2, top and middle panels). Two major species were evident with sizes corresponding to 29 and 33 kDa. Minor species were also present possessing sizes either intermediate or less than the major species. These immunoreactive proteins specifically reacted with antibodies directed to three different domains of the PLP-A molecule but not to antipeptide antibodies specific to rat PL-II (Fig. 2, middle panel). CHO cell production of recombinant PLP-A was inducible by cadmium and maintained for at least 10 days of culture under serum-free conditions (Fig. 2, bottom panel).

Comparison of the electrophoretic behavior of CHO cell-secreted PLP-A under reduced and nonreduced conditions revealed an interesting difference (Fig. 3). Unlike our previous analysis of nonreduced PLP-A from placental cytosol or serum which migrated as high molecular weight complexes (Deb and Soares, 1990), nonreduced CHO cell-PLP-A migrated as a monomer and faster than reduced PL-P-A. The faster migration is suggestive of the presence of a large looped-disulfide bridge (see Southard and Talamanca, 1991). Examination of placental cell-secreted PLP-A generated under similar serum-free culture conditions demonstrated that monomeric behavior was not unique to nonreduced CHO cell-expressed PLP-A but also a feature common to nonreduced placental PLP-A (Fig. 3). These results further suggested a role for serum proteins in the generation of the high molecular weight PLP-A complexes.

Characterization of Purified Recombinant PLP-A—Sequential steps in the purification of the 33-kDa recombinant PLP-A species are shown in Fig. 4. Steps in the isolation included: zinc sulfate precipitation, concanavalin A affinity chromatography, gel filtration chromatography, and SDS-polyacrylamide gel electrophoresis with electrophoresis. The 29- and 33-kDa PLP-A isoforms could not be resolved by gel filtration chromatography. PLP-A-positive fractions obtained from the gel filtration column were divided into two groups: fractions 50–65 and fractions 66–75. Fractions 50–65 contained most of the 33 kDa PLP-A species plus minor high molecular weight contaminating non-PLP-A protein species (data not shown). Fractions 66–75 contained proportionately less of the 33-kDa PLP-A species but were devoid of contaminating non-PLP-A protein species (Fig. 4, lane C). The fraction 66–75 pool of PLP-A was used for assessing PLP-A biological and binding activities. PLP-A isoforms electrophoresed following gel electrophoresis were used for amino acid sequencing and antibody generation.

N-terminal sequence analysis of the purified 29- and 33-kDa proteins confirmed their identity with PLP-A. The first six N-terminal amino acids were the same for both the 29- and 33-kDa species and were identical to the N terminus predicted from the PLP-A nucleotide sequence (Duckworth et al., 1986) Met1-Arg2-Ala3-Lys4-Leu5-Leu6. A minor PLP-A species (less than 20%) was also present in both the purified 29 and 33 kDa preparations. The minor species represented a truncation of PLP-A with its N terminus beginning at Leu4: Leu2-Leu6-Asn7-Val8-His9-Asn10. Verification of the N termini of PLP-A species isolated from placental tissues has not been successful (Deb and Soares, 1990).

Recombinant PLP-A did not exhibit PRL-like activities in the Nb2 lymphoma cell proliferation assay (Table I) nor GH-like activities in the 3T3-F442A adipocyte conversion assay (Table II).

Antibodies generated to the 33-kDa recombinant PLP-A specifically recognized both 29- and 33-kDa PLP-A species secreted by placental explants (Fig. 5). Furthermore, the antibodies directed to recombinant PLP-A localized PLP-A predominantly to the cytoplasm of PLP-A-secreting CHO cells and to trophoblast cells within the junctional zone of the chorioallantoco placenta (Fig. 6). Antibodies made to the 33-kDa PLP-A isoforms precipitated immunoreactive proteins specifically reacted with antibodies directed to three different domains of the PLP-A molecule but not to antipeptide antibodies specific to rat PL-II (Fig. 2, middle panel). CHO cell production of recombinant PLP-A was inducible by cadmium and maintained for at least 10 days of culture under serum-free conditions (Fig. 2, bottom panel).
in triplicate. Approximate molecular weights: PLP-A, 29-33,000; PRL, 24,000.

<table>
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<th>Treatment</th>
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<tr>
<td>PLP-A (ng/ml)</td>
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</tr>
<tr>
<td>0.1</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>1.0</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>10.0</td>
<td>6.4 ± 0.8</td>
</tr>
<tr>
<td>100.0</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>1000.0</td>
<td>6.6 ± 0.9</td>
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<tr>
<td>PRL (ng/ml)</td>
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<tr>
<td>0.1</td>
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</tr>
<tr>
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<tr>
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</table>

$^a$ Glycerol phosphate dehydrogenase activity (units/mg protein).

Data in this table are from a representative experiment performed in triplicate. Approximate molecular weights: PLP-A, 29,000-33,000; PRL, 22,000. The 1000 ng/ml concentration of GH provides maximal stimulation in the assay.

### Table II

### Effects of recombinant PLP-A and bovine GH on adipocyte differentiation of 3T3-F442A cells (mean ± S.E.)

Data in this table are from a representative experiment performed in triplicate. Approximate molecular weights: PLP-A, 29,000-33,000; PRL, 22,000. The 1000 ng/ml concentration of GH provides maximal stimulation in the assay.

<table>
<thead>
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<th>Treatment</th>
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</tr>
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<tbody>
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<td>1</td>
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<td>100</td>
<td>790 ± 49</td>
</tr>
<tr>
<td>1000</td>
<td>1192 ± 115</td>
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</table>

$^a$ Glycerol phosphate dehydrogenase activity (units/mg protein).

### Fig. 5. Characterization of antibodies generated to recombinant PLP-A. Medium conditioned by placental explants was concentrated and separated in 12.5% gels and examined by Western blot analysis using antibodies to amino acids 152-164 of the PLP-A sequence (lanes A and B) or to recombinant PLP-A (lanes C and D). Antibodies used in blots shown in lanes B and D were saturated with their respective antigens. M$\text{r}$ standards ($\times 10^7$) are shown.

kDa PLP-A protein exhibited a greater reactivity to PLP-A than did previously generated PLP-A antipeptide antibodies (Deb et al., 1989).

Identification of PLP-A Serum-binding Proteins—Serum PLP-A complexes are resistant to various perturbations such as denaturants, heat, salt concentration, pH (Deb and Soares, 1990). PLP-A monomers can be isolated from serum by the combined treatment with SDS, reducing agents, and heating (Deb and Soares, 1990). This strong association of serum PLP-A complexes permitted the use of SDS-gel electrophoresis under nonreducing conditions for the isolation of the PLP-A complexes. High molecular weight serum PLP-A complexes were enriched by concanavalin A and Affi-Gel Blue affinity chromatographies, electrophoretically separated under nonreducing conditions, transferred to nitrocellulose, and probed with antibodies generated to recombinant PLP-A.
Western blot analysis specifically identified two major PLP-A immunoreactive species possessing molecular masses of 110 and 230 kDa (Fig. 7).

In order to further determine the identity of the high molecular weight PLP-A complexes present in serum, cross-linking experiments were performed with radioiodinated recombinant PLP-A and serum collected from rats at day 19 of pregnancy. The specificity of PLP-A associated with complexes possessing sizes of approximately 130 and 250 kDa was demonstrated (Fig. 8, top panel). The migration of these two major PLP-A-serum protein complexes and some minor complexes were further resolved following immunoprecipitation with antibodies to recombinant PLP-A (Fig. 8, middle panel). Electrophoretic migrations of the major complexes under reducing conditions were at 130 and 250 kDa and the minor complexes at 70 and 110 kDa (Fig. 8, middle panel). Under nonreducing conditions the major complexes migrate faster, 110 and 230 kDa (data not shown), corresponding to the complexes identified by Western blot analysis. The specificity of PLP-A cross-linking with serum proteins was further determined by coincubation with excess ovine PRL, bovine GH, and human PL (Fig. 8, bottom panel). These hormones failed to interfere with the binding of radiolabeled recombinant PLP-A to serum proteins (Fig. 8, bottom panel) as did the addition of α₂-macroglobulin and mannose 6-phosphate (data not shown). Furthermore, immunoprecipitation with antibodies to α₂-macroglobulin failed to precipitate the PLP-A complexes (data not shown). PLP-A-binding proteins were present in serum harvested from male and female rats (Fig. 9, top panel). The concentration of the 250-kDa PLP-A-binding protein appeared to increase as gestation progressed (Fig. 9, bottom panel).

**DISCUSSION**

In the present study, we have expressed PLP-A in CHO cells, characterized recombinant PLP-A proteins, and resolved in part the nature of the high molecular weight PLP-A complex present in circulation.

PLP-A proteins expressed by engineered CHO cells possessed immunochemical and biochemical characteristics similar to PLP-A produced by trophoblast cells of the rat cho-

**Fig. 7. Immunodetection of high molecular weight PLP-A complexes.** PLP-A high molecular weight complexes were partially purified via concanavalin A and Affi-Gel Blue affinity chromatographic procedures, separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, and examined by Western blot analysis using antibodies to recombinant PLP-A. Note the specificity of the immunoreactions with the two high molecular mass PLP-A complexes at 110 and 230 kDa. Under reducing conditions the two high molecular mass complexes migrate slower, 130 and 250 kDa (see Fig. 8). M, standard (x10⁻⁴) are shown.

**Fig. 8. Specificity of cross-linking of radioiodinated PLP-A with rat serum proteins.** Serum (100 µl) collected from day 19 pregnant rat was diluted with PBS (1:1 v/v) and incubated with radiolabeled PLP-A (2 x 10⁶ cpm) overnight at 4°C in the presence or absence of excess unlabeled PLP-A (top panel) or ovine PRL, bovine GH, and human PL (bottom panel). Cross-linking was performed with bis(sulfosuccinimidy) suberate. Cross-linked products were either acetone precipitated (top panel) or immunoprecipitated with PLP-A antibodies (middle and bottom panels) and separated in 7.5% SDS-polyacrylamide gel electrophoresis under reducing conditions. Gels were then dried and processed for autoradiography. M, standard (x10⁻⁴) are shown. Note the specificity of the 130- and 250-kDa radiolabeled PLP-A cross-linked complexes (top panel). Immunoprecipitation identified major PLP-A cross-linked complexes at 130 and 250 kDa and minor complexes at 70 and 110 kDa (middle panel). Unlabeled PLP-A specifically competed for binding of radiolabeled PLP-A to serum proteins (top panel). However, the addition of excess ovine PRL (lane C), bovine GH (lane D), or human PL (lane E) failed to interfere with the cross-linking of radiolabeled PLP-A to serum proteins (bottom panel). Please note that lane B of the bottom panel represents a control for the immunoprecipitation.
The present in male and female sera (top panel) and that the 250-kDa PLP-A progressed (bottom panel).

The signal within a regulatory protein dictating PRL or GH actions are not dependent upon each and every amino acid. Receptor binding and activation are dependent upon the precise organization of selected amino acids within the protein (Cunningham et al., 1990, 1991; Cunningham and Wells, 1991). Apparently, PLP-A does not possess the basic skeletal arrangement required for activation of PRL or GH receptor signaling systems. The functional relationships of members of the rat PRL/GH family are possibly more analogous to the transforming growth factor-β family which includes transforming growth factor-βs, activin, inhibin, and Mullerian inhibiting substance. Members of this family share the same basic structure and anywhere from 25 to 90% sequence identity yet some of the individual members interact with distinct receptors and possess distinct biological activities (Massague, 1990, 1992).

Identification of the nature of the circulating high molecular weight PLP-A complexes has provided some insight into the physiology of PLP-A. Circulating high molecular weight PLP-A complexes result from the specific association of PLP-A with proteins present in serum. The PLP-A complexes exist in multiple sizes suggesting the presence of different types of PLP-A-binding proteins or possibly that the different PLP-A complexes contain multiple copies of the same binding protein. Consistent with this latter point, considerable evidence is available indicating that ligands facilitate the oligomerization of binding proteins or receptors (Cunningham et al., 1991). PLP-A-binding proteins are not related to proteins previously implicated in binding members of the PRL/GH family, α2-macroglobulin (Southard and Talamantes, 1989), mannose 6-phosphate receptor (Lee and Nathans, 1988), growth hormone-binding proteins (Sadeghi et al., 1990), or prolactin-binding proteins (Postel-Vinay et al., 1991). Additionally, PLP-A-binding proteins are not restricted to pregnant female rats. They are present in serum from both males and females suggesting a more elemental role for PLP-A. At this time, it is not known whether there are extraplacental sources of PLP-A or whether PLP-A interacts with signaling systems used by other regulatory molecules. Presumably, PLP-A-binding proteins direct the delivery of PLP-A to its appropriate target tissues. Relationships have been found between serum binding proteins and receptors (Smith et al., 1989; Baumbach et al., 1989), and this may also be evident for PLP-A-binding proteins and PLP-A receptors. Consequently, characterization of serum PLP-A-binding proteins may provide some insight into the signaling system utilized by PLP-A at its target tissues.

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