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CHARACTERIZATION AND PURIFICATION OF THE RECOMBINANT RAT PROLACTIN-LIKE PROTEIN A (rPLP-A)

by

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CHARACTERIZATION AND PURIFICATION OF THE RECOMBINANT RAT PROLACTIN-LIKE PROTEIN A(rPLP-A)

BY

NANCY L. QUAN

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

This work is dedicated to:

my parents: Mrs. Jiemin Li and Mr. Jingzeng Quan

my husband Xiangwei and son Michael

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ABSTRACT

Rat prolactin-like protein-A (rPLP-A) is a prolactin-related glycoprotein secreted by rat placenta. In our attempts to understand the function and regulation of the rPLP-A during pregnancy, the rPLP-A cDNA was inserted into a eukaryotic expression vector and then introduced into Chinese hamster ovary cells. Cell lines that secreted high concentrations of rPLP-A were isolated, and this glycoprotein was purified from the conditioned medium using gel filtration and Concanavalin A affinity chromatography. Recombinant rPLP-A is very similar to placental rPLP-A in its recognition by polyclonal antiserum raised against rPLP-A peptide, its binding to Concanavalin A and its displacement of ^{125}[I] rPLP-A peptide. Structural comparison of the recombinant and placental rPLP-A by SDS-PAGE indicated that the recombinant rPLP-A comprised several proteins with molecular mass ranging from 26-33 kDa. Treatment of both native and recombinant rPLP-A proteins with tunicamycin resulted in a similar 25 kDa precursor protein, indicating different glycosylation patterns in the two systems. A double antibody radioimmunoassay was developed for rPLP-A in order to determine the serum levels of rPLP-A during pregnancy. It was found that levels of rPLP-A in serum were first detectable on day 14 and reached maximum at day 20 (220 ng/ml); day 20 amniotic fluid contained relatively higher concentrations (317 ng/ml). Ovariectomy and adrenalectomy of rats at day 16 of gestation resulted in about 2 times higher concentrations of rPLP-A and rPL-II in serum when compared with a sham operated group, indicating that

ovarian and adrenal factors may be involved in the regulation of rPLP-A as well as rPL-II. Lastly, a glutathione-S-transferase (GST)-rPLP-A fusion protein was generated and purified for use in the production of antiserum against rPLP-A protein.

LIST OF ABBREVIATIONS

aa	amino acid
ADX	adrenalectomy
ApoAI	apolipoprotein AI
bp	base pairs
bPL	bovine placental lactogen
bPLP	bovine prolactin-like protein
bPRC	bovine prolactin-related cDNA
BSA	bovine serum albumin
°C	degree centigrade
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CHO cells	chinese hamster ovary cells
ConA	concanavalin A
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	gram
GH	growth hormone

GH-BP	growth hormone binding protein
GHF-I	growth hormone factor I
GnRH	gonadotropin releasing hormone
GST	glutathione S-transferase
hCS	human chorionic somatomammotropin
HDL	high density lipoprotein
hGH-N	human growth hormone normal
hGH-V	human growth hormone variant
hPL	human placental lactogen
hr	hour
^{125}I	iodinated
IGF-I	insulin-like growth factor-I
IGF-I BP	insulin-like growth factor-I binding protein
IGF-II	insulin growth factor-II
IL	interleukin
IPTG	isopropy-ß-D-thiogalactopyranoside
kb	kilobase
L	liter
LTH	luteotropin
M	molar
$MgCl_2$	magnesium chloride

min.	minute
ml	milliliter
mRNA	messenger RNA
mPLF	mouse proliferin
mPRP	mouse proliferin related protein
NaCl	sodium chloride
ODC	ornithine decarboxylase
oPL	ovine placental lactogen
OVX	ovariectomy
PBS	phosphate-buffered saline
pit-1	pituitary specific transcriptional factor
PL	placental lactogen
PMSF	phenylmethylsulfonyl fluoride
PRL	prolactin
PRL-IF	prolactin inhibiting factor
PRL-RF	prolactin releasing factor
Rcho-1 cell line	rat choriocarcinoma-1 cell line
RIA	radioimmunoassay
RNA	ribonucleic acid
rPL-I	rat placental lactogen I
rPL-II	rat placental lactogen II

rPL-Iv	rat placental lactogen I variant
rPLP-A	rat prolactin-like protein A
rPLP-B	rat prolactin-like protein B
rPLP-C	rat prolactin-like protein C
RRA	radioreceptor assay
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TRH	thyrotropin releasing hormone
Tris	hydroxymethyl aminomethane
uCi	microcurie
ul	microliter
uM	micromolar
UV	ultraviolet
VIP	vasoactive intestinal polypeptide
vol/wt	volume per weight
%	percent

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INTRODUCTION

I. Introduction

Pregnancy is a complex interaction between the mother and the fetus, with the successful outcome being the birth of a live and healthy fetus and the ability of the mother to provide nourishment for the fetus. It has long been known that prolactin (PRL) is an important hormone in pregnancy, particularly in its role to prepare the mammary gland for lactation. Yet in many mammalian species, excluding man and other primates, pituitary PRL levels are known to decline to very low levels at midpregnancy (Shiu et. al., 1973), while the corpus luteum continues to function, and the mammary gland continues to develop and gain the capacity to secrete milk. Therefore, it has been postulated that the placental members of the PRL family found in those species might replace the functions of PRL during late pregnancy in these species (Soares et. al., 1991; Friesen et. al., 1993).

The PRL family which includes PRL, growth hormone and placental lactogens, is a family of proteins that show structural homology with pituitary PRL. The structural similarity includes similar positioning of cysteine and tryptophan residues and additional amino acid sequence homologies. As early as 1938, Astwood and Greep reported that a rat could be hypophysectomized at midpregnancy without compromising the course of pregnancy and the birth of healthy young, and suggested that placenta could secret proteins which could take

over the functions of the pituitary during midpregnancy (Astwood and Greep, 1938). With the advent of the radioreceptor assays (RRA) using mammary gland receptors to PRL (Shiu et. al., 1973) and liver receptors to GH (Tsushima and Friesen, 1973), placental lactogens (PLs) were identified and isolated in many species during the 1970's (Kelly et. al., 1976). The RRA and Nb2 lymphoma cell bioassay (Tananka et al., 1980) have also revealed that the rat (Robertson et. al., 1982), mouse (Colosi et. al., 1982), and hamster (Southard et. al., 1987) have two placental lactogens which appear at specific times in early (PL-I) or mid-to-late pregnancy (PL-II). The complementary DNA (cDNA) cloning and sequencing of the placental lactogens from rat (Duckworth et. al., 1986a; Robertson et. al., 1990), mouse (Colosi et. al., 1987; Jackson et. al., 1986), cow (Schuler et. al., 1988), sheep (Colosi et. al., 1989) and hamster (Southard et. al., 1989) have shown that they are structurally more closely related to PRL than to GH, although PLs are more closely related to GH than to PRL in human and other primates (Niall et. al., 1971; Shine et. al., 1977).

With the development of recombinant DNA technology, additional members of the PRL family were found to be produced by placenta of rat, mouse and cow. These include the rat prolactin-like protein A (rPLP-A) (Duckworth et. al., 1986b), rPLP-B (Duckworth et. al., 1988) and rPLP-C (Deb et. al., 1991c; Deb et. al., 1991b), rat placental lactogen I variant (rPL-Iv) (Robertson et. al., 1991; Deb et. al., 1991a), the mouse proliferin (mPLF) (Linzer and Nathans, 1984; Linzer et. al., 1985), mouse proliferin-related protein (mPRP) (Linzer and Nathans, 1985), bovine prolactin-related cDNA I (bPRC-I), bPRC-II, bPRC-III (Kessler et. al., 1989) and bovine prolactin-like protein I (bPLP-I), bPLP-II, bPLP-III and bPLP-IV

(Yamakawa et. al., 1990). These PRL-related proteins are also structurally more closely related to PRL than to GH, and they are expressed according to a specific temporal and cellular patterns in these species during pregnancy.

Although the functions of PRL-related proteins remain mostly unclear, a number of functions have been proposed for this hormone family. It has been postulated that they might have important roles in the development of mammary gland, in the regulation of the secretion of the hormones required for the maintenance of pregnancy, in ensuring an adequate nutritional environment for fetal growth, as well as functioning as autocrine or paracrine growth factors for the fetus and placenta (reviewed by Ogren and Talamantes, 1987 and Soares et. al., 1991).

II. Temporal, tissue and cell-specific expression of the multiple members of the PRL family during pregnancy

In order to proceed with a discussion of the PRL family of hormones and pregnancy it is necessary to introduce terminology relevant to the morphology of the rat uteroplacental unit. The rodent possesses two placental structures: 1) the choriovitelline placenta and 2) the chorioallantoic placenta. The choriovitelline placenta develops first and degenerates by day 14 of gestation, whereas the chorioallantoic placenta develops from day 14 into two regions: 1) basal zone and 2) labyrinth zone. The basal zone is located adjacent to the decidua basalis and contains trophoblast cells and maternal vascular channels but is devoid of fetal vessels,

while the labyrinth zone is located adjacent to the developing embryo and contains trophoblast cells, maternal vascular channels and fetal vessels.

During pregnancy, the pituitary, uterus and placenta all participate in the synthesis of various members of the PRL family of protein hormones. PRL, GH and PLs are known to show structural and functional similarities and are considered to have evolved from a common ancestral gene (Chen et. al., 1989). The PLs are expressed in the placenta, and they possess activities similar to those of GHs or PRLs. In humans, hPL is more closely related to GH, sharing about 85% homology at the amino acid level and 92% at the nucleotide level (Miller and Eberhardt, 1983). In other mammals, such as ruminants and rodents, however, PLs are more closely related to PRLs than to GHs when compared within the same species. As mentioned, recent developments in molecular biology have also revealed several other proteins also related to PRL. Each of these proteins is expressed in a distinct temporal, tissue and cell-specific pattern during pregnancy. A comparison of the PRL family in human, rat and mouse is shown in Table 1. where all sequences comparison are based on amino acid level and compared with PRL and GH of the same species.

Table 1. Comparison between members of the PRL family in human, rat and mouse.

	RNA (Kb)	Protein (kDa)	similarity PRL	similarity GH	Tissue	Cell Type/Region
hPRL	1.0	22K/24K	100%	32%	Pit.	lactotrophs/somatolactotrophs/decidual
hPL	1.0	22.5K	21%	85%	plac.	syncytiotrophoblasts
rPLI	1.0	36-40K	38%	21%	plac.	giant cells/basal
rPLII	1.0	25K	35%	19%	plac.	giant cells/basal/labyrinth
rPLPA	1.0	29K,33K	45%	29%	plac.	spongiotrophoblasts and giant cells/basal
rPLPB	0.9	26-30K	44%	31%	plac.	spongiotrophoblasts/basal
	1.2	26-30K			deci.	antimesometrial region
rPLIv	1.0	29K,33K	32%	21%	plac.	spongiotrophoblasts and giant cells/basal
rPLPC	1.0	25K,29K	25%	19%	plac.	spongiotrophoblasts and giant cells/basal
LTH	?	28K	?	?	deci.	antimesometrial region
mPLI	1.0	29-42K	30%	21%	plac.	giant cells/basal
mPLII	1.0	22K	31%	31%	plac.	giant cell/basal/labyrinth
mPLF	1.0	35K,27K	31%	18%	plac.	giant cells/basal
mPRP	1.0	36-40K	39%	25%	plac.	spongiotrophoblasts/basal
rGH-like		25-30K	?	?	plac.	?

pit., pituitary; plac., placenta; deci., decidual; LTH, decidual luteotropin.

A. Anterior pituitary

PRL is secreted by lactotrophs and somatolactotrophs of anterior pituitary. The pituitary PRL from various species possesses significant structural and functional similarities (Nicoll et. al., 1986). Its functions in mammals include stimulation of the growth and development of mammary gland, stimulation of milk protein synthesis and lactation, regulation of intermediary metabolism, immunoregulation and osmoregulation between maternal and fetal compartments as well as functioning as autocrine and paracrine growth factors (Campbell et. al., 1989; Soares et. al., 1991). However, the presence of pituitary PRL after day 6 of gestation in rodents is not required for maintenance of corpus luteum (Morishige and Rothchild, 1974), suggesting the involvement of other members of PRL family as gestation advances.

Fetal pituitary PRL mRNA expression is initiated in the rat between day 17 and 18 of gestation. However, PRL protein does not increase appreciably in circulation until after parturition (Tong et. al., 1989; Chatelain et. al., 1979).

B. Uterus

Decidual PRL has been identified in primates and is indistinguishable from pituitary PRL in its chemical, biological and immunological properties (Hwang et. al., 1974; Golander et. al., 1978; Golander et. al., 1979). It is now clear that decidual PRL is produced in the decidualized endometrium during pregnancy, as well as in the normal nonpregnant endometrium during the luteal phase of the menstrual cycle (Kauma and Shapiro, 1986).

Although its role is only poorly understood, decidual PRL seems involved in the process of implantation and development of the fetus and accounts for high concentration of PRL in amniotic fluid (Golander et. al., 1978). The mRNA encoding decidual PRL is structurally different from its pituitary counterpart due to the use of a different transcription start site located 5-7 kilobases (kb) upstream of the pituitary start site (DiMattia et. al., 1990). This might explain the differences in the regulation of pituitary and decidual PRL synthesis and secretion in the human.

In the rat, a protein has been identified which binds to PRL receptors and has functional similarities to PRL itself, including stimulation of steroidogenesis in the corpus luteum: it appears to be immunologically distinct (Herz et. al., 1986; Jayatilak et. al., 1985). This 28 kDa protein which has been called decidual luteotropin (dLTH), is synthesized and secreted in the decidual tissue. Its local production has been demonstrated by *in vitro* translation of mRNA from pseudopregnant antimesometrial decidual tissue, and mRNA level decreases with gestational age (Jayatilak et. al., 1985; Jayatilak et. al., 1989). To date the structure of the protein for decidual luteotropin is not known.

Rat prolactin like protein B (rPLP-B), a member of the PRL family, apart from its expression in rat spongiotrophoblast cells, is also expressed in antimesometrial decidual tissue at day 9 to 13 of pregnancy and day 7 to day 10 of pseudopregnancy . A partial cDNA clone isolated from a day 9 deciduoma library shows identical sequences to the placental rPLP-B (Croze et. al., 1990).

C. Placenta

1. Placental PRL family

It has been over 50 years since Astwood and Greep (1938) first identified a lactogenic/luteotropic substance secreted by the midpregnant rat placenta. They and other investigators observed that the removal of the anterior pituitary in the rat or mouse after midgestation was compatible with the continuation of pregnancy (Astwood and Greep, 1938; Newton and Beck, 1939; Pencharz and Long, 1931). The corpus luteum continued to function, and the mammary gland continued to develop and gain the capacity for milk production in the absence of the anterior pituitary. Placental extracts from day 10 to 14 of gestation were shown to contain lactogenic/luteotropic activity.

The development of the radioreceptor assay (RRA) for lactogenic hormones (Shiu et. al., 1973) and the subsequent development of a sensitive *in vitro* bioassay for lactogenic hormones-the Nb2 lymphoma cell bioassay (Tanaka et. al., 1980), have led to the isolation, purification and characterization of placental lactogens (PLs) from many different species, including human, rat, mouse, hamster, bovine, sheep and other species (Ogren and Talamantes, 1988). Contrary to the early studies, Friesen and co-workers (Shiu et al., 1973; Robertson and Friesen, 1981) showed that there were actually two peaks of lactogenic activities in the pregnant rat serum: a midpregnant activity from day 8 to 12 (rPL-I) and a mid-to-late activity from day 12 to term (rPL-II). Subsequently, they established that rPL-I and rPL-II differed in molecular weight (rPL-I is a secreted glycoprotein of 36-42 kDa,

whereas rPL-II is not a glycoprotein and is secreted as a 22 kDa protein), half-time disappearance rate (20 minutes for rPL-I versus 1.2 minutes for rPL-II) as well as antigenic determinants, suggesting that the two proteins were structurally different (Robertson et. al., 1982). The cDNAs for rPL-I and rPL-II have been isolated and characterized (Robertson et. al., 1990; Duckworth et. al., 1986a). Both the cDNAs and the proteins they encoded show significant sequence similarities to each other and to other members of the PRL family but clearly distinct as well. The sequences of PLs are known for mouse PL-I and PL-II, hamster PL-I and PL-II, bovine PL and ovine PL (Soares et. al., 1991), and all of these proteins are structurally close related to PRL than to GH.

In situ hybridization studies show that rPL-I is expressed in trophoblast giant cells of junctional zone from day 8 to 12 while rPL-II is expressed in the same cells from day 11 to 16, and toward the end of the gestation, rPL-II is also expressed in the trophoblast giant cells of the labyrinth zone (Duckworth et. al., 1990; Faria et. al., 1990a). Since labyrinth zone is located adjacent to the developing embryo and contains both maternal and fetal blood vessels, rPL-II might have access to the fetal blood supply and have a role in fetal development. This is only a speculation now, although there has been a report that there might be a mPL-II specific receptor in the fetal mouse liver (Harigaya et. al., 1988).

During the cloning of the rPL-II cDNA, two additional PRL-related cDNAs from developing placenta were discovered and further characterized. These were named rat prolactin-like protein A (rPLP-A) and rPLP-B because of their sequence similarities with PRL. The rPLP-A has 45% homology with rPRL while only 29% with rGH (Duckworth et.

al., 1986b), and rPLP-B has 44% homology with rPRL and 31% with rGH (Duckworth et. al., 1988). The rPLP-A is a glycoprotein synthesized mainly as two distinct molecular weight species (29 kDa and 33 kDa) in placenta and rPLP-B is also a glycoprotein, ranging from 26-30 kDa (Deb et. al., 1989; Ogilvie et. al., 1990b). Immunocytochemistry and *in situ* hybridization experiments show that rPLP-A is expressed in day 14 to term spongiotrophoblast cells and trophoblast giant cells in junctional zone while rPLP-B mRNA is found in day 14 to term spongiotrophoblast cells in the junctional zone (Duckworth et. al., 1990; Campbell et. al., 1989)

Another member of the placental PRL family closely related to rPL-I, termed rPL-I variant (rPL-Iv), has also been identified. When a cDNA clone for rPL-I was hybridized to a Northern blot of placental RNA isolated from day 8 to 21 placenta, a strong expression of rPL-I mRNA was observed in day 8 to 12 placenta and a faint hybridization also occurred in day 16 to term placenta, which persisted even under highly stringent hybridization and washing conditions (Robertson et. al., 1990). The cDNA for rPL-Iv was isolated and sequenced, revealing a sequence closely related to rPL-I (88% sequence identity) but clearly distinct as well. The rPL-Iv mRNA has been identified in the spongiotrophoblast cells and trophoblast giant cells from day 15 to term rat placenta (Robertson et. al., 1991). Independent work by Soares and co-workers has also led to the discovery of rPL-Iv during the purification of rPLP-A from placental explants (Deb et. al., 1991a). A major contaminant was isolated and shown to possess extensive amino acid identity to rPL-I (39 of 45 N-terminal amino acids were identical). The rPL-Iv is a glycoprotein of 29 and 33 kDa secreted by rat placenta.

Despite its significant structural homology to rPL-I, distinct differences are also evident (Deb et al., 1991a): 1) Antibodies generated against rPL-Iv specifically recognizes rPL-Iv, not rPL-I; 2) Rat PL-Iv binds to Concanavalin A whereas rPL-I lacks affinity for Concanavalian A, suggesting different glycosylation patterns and 3) The rPL-Iv is not as lactogenic as rPL-I in the Nb2 cell bioassay (Deb et. al., 1991a).

Another major contaminant protein during purification of rPLP-A has also been purified and cloned (Deb et. al., 1991b; Deb et. al., 1991c). It was named rat PRL-like protein C (rPLP-C) based on its structural similarity with PRL and other members of the PRL family. The rPLP-C consists of two major secretory forms, a 25 kDa nonglycosylated and a 29 kDa glycosylated species. The rPLP-C mRNA was first detected between days 13 and 14 of gestation and remains elevated until term. *In situ* hybridization analysis indicated that rPLP-C mRNA was specifically expressed by spongiotrophoblast cells and some trophoblast giant cells in the junctional zone of day 14 to term rat placenta (Deb et. al., 1991c).

The genes for rPRL, rPL-II, rPLP-A, rPLP-B and rPLP-C have been localized to chromosome 17 of the rat genome whereas rGH has been localized to chromosome 10 (Cooke et. al., 1986; Levan et. al., 1991; Deb et. al., 1991c). The chromosomal location of rPL-I and rPL-Iv have not been determined. These results indicates that unlike human PL which is structurally highly related to hGH, rat placental PRL family may have arisen from rPRL by gene duplication events, similar to the human GH/PL gene cluster (Berczi et. al., 1981).

In the mouse, proliferin (mPLF) was initially cloned from the proliferating mouse 3T3

cells, which expressed a protein related to mouse PRL (Linzer and Nathans, 1984). Both mRNA and protein have been identified in placental tissue and mRNA has been localized in the trophoblast giant cells (Linzer et. al., 1985). Proliferin mRNA levels rise to a peak during midgestation and decline before parturition, corresponding to the changes in protein levels in the amniotic fluid and maternal serum (Lee et. al., 1988). The protein has 31% sequence identity with mPRL, but only 18% with mGH. It is glycosylated and secreted by placental cells and 3T3 cells in culture, therefore it is thought to be a potential autocrine growth factor (Lee and Nathans, 1987). A proliferin-related protein (mPRP) was identified from a mouse placental cDNA library (Linzer and Nathans, 1985), which had significant homology with mPLF (37%) and mPRL (39%) though less with mGH (25%). The mPRP mRNA was identified in spongiotrophoblast and giant cells of the mouse placenta. Both mPLF and mPRP are glycoproteins with significant structural similarities to pituitary PRL and are present in high concentration in maternal circulation (Lee and Nathans, 1987; Colosi et. al., 1988b). Similar to rPLP-A, rPLP-B and rPLP-C which have been identified only in the rat, mPLF and mPRP appear to be unique to the mouse.

Multiple members of the PRL family have also been reported in the placenta of the cow. These include four distinct PRL-like proteins, bovine prolactin-like protein I (bPLP-I), bPLP-II, bPLP-III and bPLP-IV which were isolated from bovine full term placenta (Yamakawa et. al., 1990) and three other PRL-related cDNA, bovine prolactin related cDNA-I (bPRC-I), bPRC-II and bPRC-III which were identified from bovine placenta of 6 month gestation (Schuler and Hurley, 1987; Kessler et. al., 1989). They all show high

sequence homology with members of the PRL family and are more closely related to PRL than to GH. The expression of these bovine PRL-related cDNA clones in developing bovine placenta are apparently tissue and stage-specific as well (Duello et. al., 1986; Kessler et. al., 1989; Yamakawa et. al., 1990).

In summary, all these placental PRL-related proteins from different species, despite their significant sequence homology with PRL, are unique proteins and appear to be synthesized in specific temporal and cellular patterns in the developing placenta of these species.

2. Placental GH family

The human placenta expresses proteins with significant homology to pituitary growth hormone (hGH) (Bewley, 1977; Kaplan and Grumbach, 1981): the major one of these is human placental lactogen (hPL), also known as human chorionic somatomammotropin (hCS). The hCS is a single protein which is encoded by two genes hCS-A and hCS-B which are identical except for one minor difference in the signal sequence coding region (Barrera Saldana et. al., 1983). The hCS genes are clustered together with the pituitary hGH gene (hGH-N) and a variant hGH gene (hGH-V), on the long arm of chromosome 17 (Owerbach et. al., 1980). Human PL has only 21% homology with PRL, but very high homology (85% homology at amino acids level) with the hGH (Shine et. al., 1977). The hormone is synthesized in the syncytiotrophoblastic villous epithelium of the placenta (Hoshina et al., 1982), and it is secreted from the first trimester through to term (Berle, 1974).

The hGH variant (hGH-V), was initially thought not to be expressed, but it is now clear that the gene is transcribed in human placental tissue (Frankenne et. al., 1987), and gives rise to two major mRNA species which code for two distinct hGH-V proteins generated by alternative splicing (Cooke et. al., 1988). Very little hGH is found in amniotic fluid, implying that the hormone is secreted only into the maternal compartment (Frankenne et. al., 1988). The biological functions of placental hGH remain unknown, but they are likely to be responsible for the increased maternal production of insulin-like growth factor I (IGF-I) during late pregnancy (Hall et. al., 1984).

In the rat, a family of four GH-related proteins with 76-97% homology to each other are synthesized and secreted by day 15 placental explants (Ogilvie et. al., 1990a). The four proteins are among the major placental proteins secreted during late pregnancy. The nature of these GH-related proteins remains to be elucidated.

D. Overview of expression of the PRL family during pregnancy

During pregnancy, rat pituitary, uterus and placenta produce many proteins related to prolactin which are expressed according to temporal, tissue and cell-specific patterns. A twice daily surges of pituitary PRL is initiated after the copulatory stimulus and continues until midgestation; the other members of the PRL family are expressed around midpregnancy. Rat PL-I, decidual luteotropin and rPLP-B are expressed in early-to-mid gestation: rPL-I is produced by trophoblast giant cells in placenta, while decidual luteotropin and rPLP-B are secretory products of antimesometrial decidua. At midpregnancy, the twice daily surges of

PRL cease whereas rPL-II is secreted by trophoblast giant cells, first by the junctional zone and followed by both the junctional and the labyrinth zone as gestation advances. Secretion of rPLP-A, rPLP-B, rPLP-C and rPL-Iv is initiated in the junctional zone around days 13 of gestation, reaches maximum level at day 18 and continues until term. Secretion of rPLP-A, rPLP-C and rPL-Iv is confined to spongiotrophoblast cells and some trophoblast giant cells of the junctional zone while rPLP-B expression is restricted to spongiotrophoblast cells of junctional zone. An overview of expression of different members of the PRL family during pregnancy in the rat is shown in Fig.1.

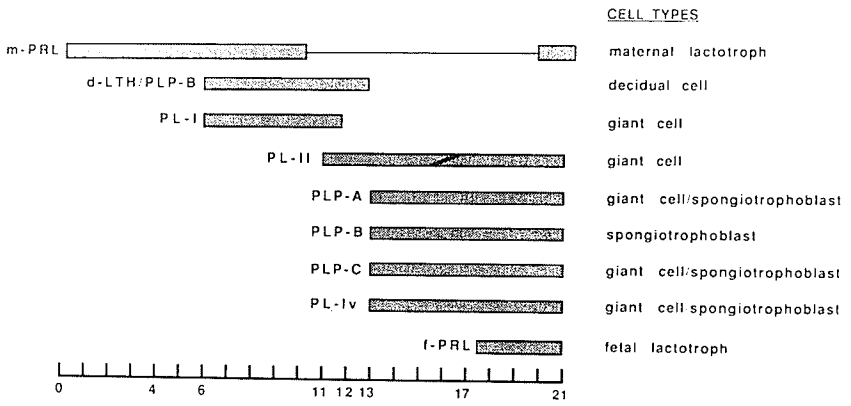


Fig. 1. Schematic diagram of the temporal and cellular pattern of expression of the PRL family during pregnancy in the rat. mPRL, maternal anterior pituitary PRL; d-LTH, decidual LTH; f-PRL, fetal anterior pituitary PRL. The scale on the bottom of the diagram refers to

the duration of gestation in the rats. Day 0, sperm positive; day 4, implantation; day 21, parturition. (Soares et. al., 1991)

III. Gestational profiles of the PRL family

The gestational profiles of PLs in the maternal circulation fall into two general patterns (Kelly et. al., 1976). The first includes the serum profiles of rat, mouse and hamster PLs, which are characterized by two peaks: one is PL-I which peaks during midpregnancy only and the second is PL-II which appears at midpregnancy and remains elevated until term. The gestational profiles of the bovine and ovine PLs are characterized by the continued presence of the hormones in the blood from the time they first appear until parturition. The species differences in the maximum PL concentrations found in maternal blood are quite marked, with values ranging from a few nanograms per millilitre of serum in the bovine to micrograms per millilitre of serum in the primates, sheep, mouse, rat and hamster (Ogren and Talamantes, 1988).

For the other members of the PRL family, mPLF and mPRP in the mouse are expressed in the midpregnant mouse placenta, first appearing at day 8, peaking at day 10 and gradually declining by day 18; while in the rat, rPLP-A, rPLP-B and rPLP-C first appear at around day 13 to 14 and remain high until term. In rodents, the pituitary PRL levels fall during midpregnancy, suggesting that the presence of multiple members of the PRL-related proteins might replace the functions of PRL during late gestation to maintain the normal

pregnancy and lactation.

Of the species that have been examined thus far, it appears that the ruminants differ from both the primates and rodents in the distribution of PLs between maternal and fetal circulations. In the sheep, the oPL concentrations in the fetal blood exceed that of the maternal blood for the first half of the pregnancy (Chan et. al., 1978) and in the bovine, the fetal serum bPL concentration is about 5 to 10 times higher than its concentration in the maternal blood throughout gestation (Duello et. al., 1986). The species differences in the distribution of PLs between fetal and maternal compartments are interesting because they suggest that the role of PLs in the fetus might be relatively more important in the species with higher fetal PL concentrations than those with lower PL concentrations.

The hPL (Grumbach et. al., 1968; Nielsen et. al., 1979), mPL-II (Colosi et. al., 1987) and mPLF (Lee et. al., 1988) have also been detected in the amniotic fluid in concentrations significantly higher than those in fetal serum. Both oPL (Chan et. al., 1978) and bPL (Duello et. al., 1986) are present in amniotic and allantoic fluids. However, the role of the PRL-related proteins in these compartments is unknown.

IV. Regulation of secretion and synthesis of the PRL family

All members of the PRL family examined to date, are homologous throughout the coding region of the mature hormone and the hydrophobic region of the signal peptide. However, the 5'-flanking region differs between pituitary hormone PRL and the PRL-related

members expressed in the placenta and decidua, which may contribute at least in part to the tissue-specific expression patterns of PRL family members (DiMattia et. al., 1990; Shida et. al., 1992).

A. Pituitary and decidual PRL

The expression of PRL is very tissue specific--the gene is present in all cells, but in the rat it is only expressed in the anterior pituitary, while in human it is also expressed at low level in the decidualized endometrium (Kauma and Shapiro, 1986) and lymphocytes (Pellegrini et. al., 1992; Lytras et. al., 1993). The synthesis and release of pituitary PRL is regulated by dopamine, thyrotropin releasing hormone (TRH), vasoactive intestinal polypeptide (VIP) and estrogen (Ben Jonathan et. al., 1989; Tong et. al., 1989). The regulation at the gene level is controlled by multiple transcription factors which include Pit-1/GHF-1 and estrogen receptor (Keech and Gutierrez Hartmann, 1989; Karin et. al., 1990; Ingraham et. al., 1990; Maurer et. al., 1990; Harvey et. al., 1991). These transcription factors possibly with other unidentified factors, bind to *cis*-acting elements present in the 5'-flanking region of the PRL gene and dedicate the cell-specific expression of PRL to the lactotrophs and somatolactotrophs of the anterior pituitary.

Human decidual tissue synthesizes and secretes a protein which is identical to pituitary PRL in its chemical, biological and immunological properties (Braverman et. al., 1984). However, the regulation of the synthesis and release of PRL from decidual tissue is different from that of pituitary PRL. The first striking difference in the regulation is that dopamine and

dopamine agonists have no inhibitory effect on decidual PRL secretion (Richards et. al., 1982) or amniotic fluid PRL levels (Healy and Hodgen, 1983). Estrogen exerts a strong stimulation on pituitary lactotrophs, but appears at most to have only small effects on decidual PRL production (Rosenberg and Bhatnagar, 1984). On the other hand, progesterone appears to stimulate decidual PRL release while having little effect on pituitary PRL production (Ying et. al., 1988). Recent studies showed that the mature decidual PRL transcript is longer than the pituitary transcript and that the regulation of transcription is very different in the two tissues. Cells from the two tissues utilize different transcriptional start sites, and as a consequence, an additional exon, located 5-7 kb upstream from the pituitary PRL mRNA starting site is included in the decidual PRL mRNA, adding a 5'-untranslated extension of approximately 150 nucleotides (DiMattia et. al., 1990). Unlike the pituitary, decidual tissue does not express the transcriptional factor Pit-1/GHF-1. It is therefore unlikely that Pit-1/GHF-1 consensus sequences present in the PRL gene would have a role in decidual prolactin mRNA regulation (DiMattia et. al., 1990).

Further studies indicated that the synthesis and release of decidual PRL are regulated by multiple factors released by placenta, fetal membranes and decidua. A decidual PRL releasing factor (PRL-RF), has been purified and found to be a 23.5 kDa protein, which stimulates an acute increase in PRL release and a secondary increase in PRL synthesis and release (Handwerger et. al., 1987a; Golander et. al., 1988). Insulin-like growth factor I (IGF-I) which is released by the placenta (Thrailkill et. al., 1988) and relaxin which is released by both placenta and decidua, stimulate a prolonged increase in the synthesis and

release of PRL beginning 48 hr after exposure. PRL inhibitory factor (PRL-IF) which is released by the decidua, and lipocortin which is released by both placenta and decidua, inhibit both basal and PRL-RF mediated PRL release (Handwerger et. al., 1991b; Handwerger et. al., 1991a). These findings suggest that there is a complex interaction between the placenta, fetal membranes and decidua in the regulation of the synthesis and secretion of decidual PRL.

B. Human placental lactogen

The mechanism involved in the regulation of secretion of hPL is not fully understood. In order to study the physiology of hPL secretion, many investigators have studied the dynamics of the synthesis and secretion of hPL in placental explants. However, these studies have shown no consistent effects of glucose, estrogen, glucocorticoids, oxytocin, prostaglandins, epinephrine, thyrotropin releasing factor (TRH), gonadotropin releasing factor (GnRH) or dopamine on the synthesis and secretion of hPL (Niven et. al., 1974; Handwerger et. al., 1973; Hershman et. al., 1973). Recent studies have shown that angiotension II (Petit et. al., 1989), Insulin-like growth factor II (IGF-II) (Bhaumick et. al., 1987) and apolipoprotein AI (ApoAI) (Handwerger et. al., 1987b) can stimulate hPL release.

Apolipoprotein AI (ApoAI), a major apoprotein constituent of high density lipoprotein (HDL), has been shown to stimulate a dose-dependent increase in hPL synthesis (Richards et. al., 1990) and release (Handwerger et. al., 1987b) from cultured trophoblast cells. In *in vivo* studies, the intravenous infusion of ApoAI into pregnant ewes has also been shown to

stimulate a significant increase in serum hPL concentration (Grandis et. al., 1989). Since both plasma ApoAI (Fahraeus et. al., 1985; Desoye et. al., 1987) and hPL (Grumbach and Kaplan, 1964) concentrations increase progressively during pregnancy, these results may suggest a novel physiological role for ApoAI in the regulation of hPL secretion.

The intracellular mechanisms involved in stimulating hPL synthesis and release are still unclear. Studies have shown that ApoAI stimulates a dose-dependent increase in adenylate cyclase activity in placental membranes and cyclic adenosine monophosphate (cAMP) production in trophoblast cells (Wu et. al., 1988). Other reports have shown that phospholipase A2 and arachidonic acid stimulate hPL release (Zeitler et. al., 1986). Although further studies are necessary to clarify the exact intracellular mechanisms involved in hPL release, studies to date clearly indicate that there is probably a complex interaction of multiple signal transduction pathways involved in hPL release.

Information on the molecular mechanisms regulating the expression of the hPL mRNA is limited compared with hPRL and hGH. Saunders and co-workers (Rogers et. al., 1986; Walker et. al., 1990; Fitzpatrick et. al., 1990; Lemaigre et. al., 1989) have used the human choriocarcinoma JEG-3 cell line to study the regulation of hPL gene expression. A transcriptional enhancer has been localized to a 138 base pairs (bp) region located 2 kilobases (Kb) 3' to the hPL-3 gene which may be at least partly responsible for trophoblast cell-specific expression of hPL and may be associated with the gestational modulation of hPL expression (Walker et. al., 1990). This region can specifically bind nuclear proteins isolated from human choriocarcinoma cell line JEG-3 or placenta. Additional putative regulatory

elements (SP1 binding sites) which are required for basal hPL expression have been found between -142 to -129 base pairs (bp) in the 5'-flanking region of the hPL-3 gene. However, these sites do not appear to regulate tissue specific expression based on functional and binding studies (Fitzpatrick et. al., 1990). Thyroid hormone receptor can bind to a region of the hPL promoter in a DNA-binding assay (Barlow et al., 1986). When introduced into a rat pituitary cell line, thyroid hormone and dexamethasome increased expression of a transient reporter gene containing 500 bp of the hPL promoter (Cattini and Eberhardt, 1987). In addition, hPL gene regulation by thyroid hormone has been recently demonstrated in a placental system as thyroid hormone increased levels of endogenous hPL mRNA and protein production in human choriocarcinoma cells (Nickle and Cattini, 1991). Recent studies showed that hGH-V and hPL mRNA levels are differentially regulated in the placental tumor cells treated with methotrexate. It was suggested that the mechanism is through modulation of thyroid hormone response of the hGH-V and hPL genes (Nickle et al., 1993).

C. Other members of the placental PRL family

Studies on the regulation of the placental PRL family in other species are still in the early stage. To examine whether any extraplacental factors influence PL secretion, a series of endocrine ablation experiments were performed to examine the potential influences of the pituitary, ovary and fetus on PL secretion. Hypophysectomy of the rat and mouse at midpregnancy resulted in significant elevation of rPL-II and mPL-II during late gestation (Robertson et. al., 1984a; Day et. al., 1986; Soares and Talamantes, 1985; Voogt et. al., 1985)

and mGH appears to be responsible for the anterior pituitary mediated decrease in serum mPL-II concentration (Kishi et. al., 1988) possibly by modulating the clearance of mPL-II (Pinon et. al., 1988). Bilateral ovariectomy of the day 14 pregnant rat led to a rapid increase in serum rPL level. Adrenalectomy combined with ovariectomy also led to sustained elevated levels of serum rPL-II which were greater than those seen with bilateral ovariectomy alone. Neither progesterone or estrogen were able to reduce serum rPL-II levels in ovariectomized animals, which suggests that some undetermined ovarian factors might influence rPL-II secretion (Robertson et. al., 1984a). Removal of fetuses at day 14 of gestation in pregnant rats led to marked suppression of serum rPL-II levels possibly because the labyrinth zone which is the main source of rPL-II in late pregnancy does not develop normally (Robertson et. al., 1984b). It is not known whether pituitary, ovary and fetal hormones act directly or if the effects are secondary, possibly through metabolic changes.

A decidual protein has been purified and characterized which was found to stimulate mPL-II release. It is the mouse homologue of the calcium binding protein, calcyclin (Guo et. al., 1990), and therefore named mouse decidual calcyclin (Thordarson et. al., 1991). The purified decidual calcyclin stimulates the release of mPL-II from cultured trophoblast cells in a dose-dependent manner at concentrations from 0.01 to 1 ug/ml. The mechanism by which decidual calcyclin affects the release of mPL-II is not known, however, it might exert its function though affecting the availability of calcium (Thordarson et. al., 1991).

In a recent experiment utilizing transgenic mice, a region extending from -2700 to -256 base pairs (bp) of the 5'-flanking region in the mPL-II gene has shown to be responsible

for trophoblast cell-specific mPL-II expression (Shida et. al., 1992). However, it will be necessary to narrow down the region which is responsible for tissue and cell-specific expression and also compare the sequence with other members of the family across species.

In mPLF, a phorbol ester responsive element and a negative glucocorticoid regulatory elements (GREs) have been identified (Connor et. al., 1989; Mordacq and Linzer, 1989). Structural analysis of the 5'-flanking region of genes for bPL and bPRC-I, have indicated the presence of consensus sequence for Pit-1/GHF-1, thyroid hormone and phorbol ester responsiveness (Ebbitt et. al., 1989; Kessler and Schuler, 1991). Whether the presence of these consensus sequences denotes any functional control remains to be determined. The mechanisms that regulate other members of the PRL family are essentially unknown.

The studies on the regulation of the placental PRL family are still at their early stage, both at the cellular and molecular levels. The discovery that the rat choriocarcinoma Rcho-1 cell line expresses members of the rat placental PRL family, including rPL-I, rPL-II, rPLP-A, rPLP-C and rPL-Iv (Faria et. al., 1990b; Faria and Soares, 1991), will be useful in studying the regulation of the synthesis and release of the hormones. The cell line could also prove useful in characterizing the regulatory elements of the members of the rat placental PRL family and possibly in identifying the mechanisms controlling trophoblast-specific gene expression.

V. Receptors for the PRL family

A. The expanded family of PRL/GH/cytokine receptors

The recent identification of the cDNAs encoding PRL and GH receptors has led to the discovery that their receptors form a gene family as well, which has been called cytokine receptor superfamily (Kelly et. al., 1991). This family include the receptors for PRL (Boutin et. al., 1988), GH (Leung et. al., 1987), erythropoietin (D'Andrea et. al., 1989), interleukin 2 (IL2) (Hatakeyama et. al., 1989), IL3 (Itoh et. al., 1990), IL4 (Mosley et. al., 1989), IL5 (Takaki et. al., 1990), IL6 (Yamasaki et. al., 1988), IL7 (Goodwin et. al., 1990), granulocyte and macrophage-colony stimulating factor (GM-CSF) (Gearing et. al., 1989) and granulocyte-colony stimulating factor (G-CSF) (Fukunaga et. al., 1990). These receptors possess a single hydrophobic transmembrane domain, a highly variable cytoplasmic domain (both in length and sequence) and an extracelluar domain. Members of this family show significant sequence similarity in their extracelluar ligand-binding domain (Bazan, 1989). The ligands for these receptors are structurally unrelated except for PRL and GH. However, they all share a common feature of having multiple biological actions (Bazan, 1989).

In the extracelluar domain, there are two characteristic features that are consistent throughout all members of the family. The first is the presence of two pairs of cysteine residues, usually in the amino-terminal region of the molecule (Bazan, 1990). These cysteine residues may be involved in forming ligand binding pockets characteristic for each specific ligand. The second feature is a highly conserved WSXWS motif (tryptophan, serine, any

amino acid, tryptophan, serine) near the carboxyl-terminal extremity of the extracelluar domain, is found in all members of the family except the GH receptors in which there are conservative substitutions (Thoreau et. al., 1991). This domain may contribute to the interaction of receptors with their ligand or with other components of the receptor complex.

B. PRL/GH receptors

The PRL receptors have been cloned and shown to consist of related members which differ mostly in their cytoplasmic domain (Shirota et. al., 1990; Zhang et. al., 1990). Unlike in the human and rabbit where only a single form of PRL receptor has been identified, there are multiple forms of the PRL receptors in the rat. The PRL receptor cloned from the rat liver (short form), contains 291 amino acids with a short cytoplasmic domain of 57 amino acids (Boutin et. al., 1988). Using the cDNA of the short form of the receptor as a probe, a cDNA library prepared from rat ovary was screened and a cDNA was identified which encodes a mature receptor of 592 amino acids (long form), with extracellular and transmembrane regions that are very similar to those of rat liver PRL receptor but an intracellular domain much longer (358 amino acids) than that found for rat liver (Edery et. al., 1989). Recently, a mutant form of PRL receptor was identified in the rat Nb2 lymphoma cell line. The Nb2 lymphoma cell line is dependent on PRL for growth and contains high affinity PRL receptors (Tanaka et. al., 1980; Shiu et. al., 1983). The PRL receptor cDNA cloned from a Nb2 lymphoma cell cDNA library encodes a novel form of PRL receptor (intermediate form). This receptor is structurally similar to the short and long forms of the PRL receptors in the

extracellular and transmembrane domain; however, its cytoplasmic domain is missing 198 amino acids when compared with the long form of the PRL receptor. This structures appears to be due to a mutation in the PRL receptor gene, resulting in a loss of 594 nucleotides in a region encoding a major portion of the cytoplasmic domain of the long form of PRL receptor (Ali et. al., 1991).

The GH receptors have been cloned for human, rabbit, rat, mouse, cow and sheep. These receptors share approximately 70% overall sequence similarity. Several studies have demonstrated multiple forms of GH receptors in mouse (Smith and Talamantes, 1987) and human liver (Hocquette et. al., 1990). Recent studies have shown that a tissue-specific hGH receptor mRNA with an exon 3 deletion is presented in placental tissue and cell lines. Since exon 3 included a segment in the extracellular domain of the receptor, its alternative inclusion or exclusion may mediate critical alternations in hormone binding and physiological functions (Urbanek et. al., 1992).

A GH binding protein (GH-BP), identified in mouse (Peeters and Friesen, 1977), rabbit (Ymer and Herington, 1985; Ymer and Herington, 1987) and rat serum (Baumbach et. al., 1989) is a soluble, short form of the liver GH receptor (Sadeghi et. al., 1990). There is amino acids identity of the amino-terminal sequences of the GH receptor. The production of the GH-BP appears to be due to either specific proteolytic cleavage of the membrane form of the receptor (in man, cow and rabbit) or alternate splicing processing of the GH receptor mRNA (in rat and mouse) (Baumann et. al., 1987; Daughaday et. al., 1987; Sadeghi et. al., 1990).

The comparative structures of PRL and GH receptors are summarized in Fig.2. Short and long forms of receptors exist for both hormones. For PRL, the short form is membrane bound, whereas for GH the short form is a soluble binding protein. Although in general, the amino acid identity is approximately 30% between PRL and GH receptors, this increases to about 70% in certain domains of the extracelluar regions, especially in the region between the first two pairs of cysteines, and in the cytoplasmic domain just inside the transmembrane segment. In addition, there are other extracellular and cytoplasmic regions of moderate identity (40-60%). This high structural identity has led to the conclusion that the receptors for PRL, GH and other growth factors form a new family of single membrane-spanning receptors (Boutin et. al., 1988). A schematic comparisons of the PRL and GH receptors are shown in Fig. 2.

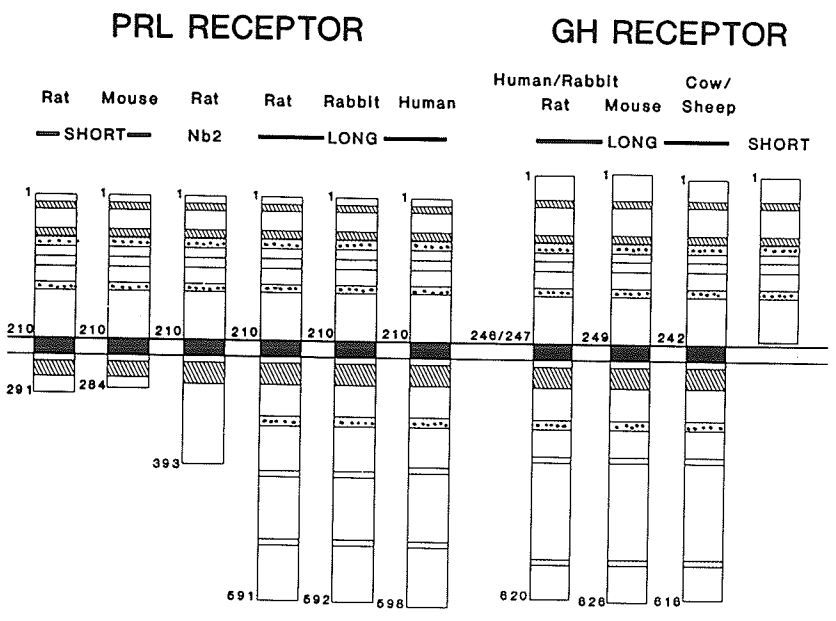


Fig. 2. Schematic representation of the PRL/GH receptor family. The short form of the PRL receptor from rat and mouse, the intermediate form from Nb2 cells, and the long form of the receptor in rat, rabbit and human are compared to long and short (binding protein) forms of GH receptor in human, rabbit, cow, sheep, mouse and rat. The first and last amino acid of the mature proteins are indicated, as well as the last amino acid of the extracellular domain. The transmembrane domains are shown in black. Regions of increased (>68%) amino acid identity are cross-hatched, and moderate (40-60%) identity are stippled. (Kelly et al., 1991).

C. Receptors for PLs

Previous studies of the binding of PLs to tissues of postnatal rabbit and rat suggested that the biological actions of the hormones were mediated through binding to PRL and/or GH receptors (Shiu et. al., 1973; Tsushima and Friesen, 1973). Studies using receptor cross-linking experiments and radioreceptor assays, suggested, however, that the biological actions of hPL and oPL in the human and ovine fetus were mediated through binding to a distinct and species specific PL receptor (Freemark and Handwerger, 1986; Freemark et. al., 1986; Freemark et. al., 1987; Freemark et. al., 1992). The ovine PL receptor has been purified and found that it is a 38-47 kDa protein which has high affinity for oPL and low affinity for oGH and oPRL (Freemark et. al., 1988; Freemark and Comer, 1989). Specific hPL receptors have been detected in human fetal tissues (Hill et. al., 1988), and oPL receptors have been reported in the liver of the fetal lamb and pregnant sheep (Freemark and Handwerger, 1986; Freemark et. al., 1988). Although specific PL receptors have not been cloned yet, it has been

postulated that the PL, GH and PRL receptors constitute a family of protein receptors which differ in their relative affinities for PLs, GHs and PRLs (Freemark et. al., 1988; Freemark et. al., 1992).

There are also striking differences in the expression of the PL, GH and PRL receptors during ontogeny. Specific PL binding sites are detected in the liver of the fetal lamb (Freemark and Handwerger, 1986; Freemark et. al., 1988) and fetal mouse (Harigaya et. al., 1988) as well as in the liver and skeletal muscle of the human fetus (Hill et. al., 1988) as early as midgestation when homologous sources of PLs are used in the experiments. In contrast, there is little or no specific binding of radiolabelled GH or PRL to human fetal skeletal muscle or to tissues of the fetal rat, rabbit, rhesus monkey, mouse or sheep. During the latter half of gestation, the number of hepatic oPL receptors in the ovine fetus increases markedly, reaching a peak at 3-7 days before parturition (Freemark et. al., 1986). The demonstration that fetal and maternal tissues contain PLs receptors which are structurally and functionally distinct from GHs and PRLs receptors provides a strong support for PLs in the regulation of fetal and maternal metabolism (Freemark et. al., 1989; Freemark et. al., 1990).

The specific receptor for bovine PL has also been reported (Galosy et. al., 1991). The specific receptors for bPL has been detected in the endometrial membranes isolated from uterus of midpregnant cow. Both bGH and bPL competed with [^{125}I] bPL for binding, however, the concentration of bGH needed for effective competition was 100 fold higher than those required for bPL. No specific binding of radiolabeled bGH was detected in endometrial tissue suggesting the absence of bGH receptors in endometrium. The presence of distinct PL

receptors in the endometrium of midpregnant cows suggests possible roles for bPL in the maintenance of pregnancy (Galosy et. al., 1991).

Studies of the biological effects of PL, GH and PRL in other mammalian fetal tissue when using nonhomologous source of PLs, also suggested the presence of distinct fetal PL binding sites. For example, oPL, but not GH and PRL, stimulated ornithine decarboxylase (ODC) in fetal rat liver (Hurley et. al., 1977), IGF-II production by rat fibroblasts (Adams et. al., 1983), amino acids transport in fetal rat diaphragm (Freemark and Handwerger, 1984) glycogen synthesis in fetal rat hepatocytes (Freemark and Handwerger, 1984), . However, the binding of the rPL to homologous fetal tissue has not yet been examined and there may be species differences in the specificities of fetal receptors for the various PLs, GHs and PRLs. Harigaya and co-workers have compared the binding of mPL-II and mPRL to liver membrane in the pregnant mouse (Harigaya et. al., 1988). They found that the affinity and number of binding sites for mPL-II was always higher than those of mPRL during pregnancy. The molecular weight of the receptor estimated by receptor cross-linking experiment was 45 kDa for mPL-II and 40 kDa for mPRL. These results suggested that a unique mPL receptor might exist in pregnant mouse as well (Harigaya et. al., 1988).

D. Receptors for other members of the PRL family

Other members of the PRL family, mPLF and mPRP in mouse, rPLP-A and rPLP-C in rat, have shown no lactogenic activity in the Nb2 cell bioassay (Lee et. al., 1988; Deb and Soares, 1990; Deb et. al., 1991b), suggesting they might not act through the PRL receptor,

but might have specific receptors. Recombinant mPLF purified from Chinese Hamster Ovary (CHO) cells was shown to bind to the mannose-6-phosphate receptor (Lee and Nathans, 1988), but the nonglycosylated mPLF did not compete with the glycosylated mPLF, indicating that the binding might be mediated through a carbohydrate group (Lee and Nathans, 1988). Furthermore, mPLF secreted by mouse placental tissue which is also glycosylated, competed poorly with the glycosylated mPLF produced by CHO cells (Lee and Nathans, 1988). Therefore, whether native mPLF protein binds to the mannose-6-phosphate receptor is still questionable. There is no information about the nature of the receptors to date for the other lactogenic and nonlactogenic placental PRL-related proteins,

VI. Biological actions of the PRL family

The development of the embryo/fetus in the female reproductive tract necessitates significant adjustments in the functions of maternal tissues. As suggested above, members of the PRL family are at least part of the signalling system utilized during pregnancy to ensure coordinated responses in maternal and fetal compartments. Pituitary PRLs and PLs are functional analogs. The rPLP-A, rPLP-B and rPLP-C in rat, mPLF and mPRP in mouse are structural analogs of pituitary PRL, whose biological actions are yet to be determined (Soares et. al., 1991). Each member of the placental PRL family is a major secretory product of the placenta, suggesting that they might be functionally significant during pregnancy.

The functions of PRL are not well understood in mammals. We know that PRL is a

multifunctional protein with pronounced physiological effects on growth, reproduction and osmoregulation across species. PRL receptors are found in many different organs including mammary gland, liver, kidney, brain, prostate, testis and ovary (Kelly et. al., 1991). Since in rodents PRL levels fall during midpregnancy, it is possible that these functions are being carried out by a set of unique PRL-related proteins rather than by PRL itself.

Since PLs are structurally similar to PRLs and GHs, most of the work carried out on the biological actions of the PLs have examined the PRL- and GH-like effects of these hormones in various tissues. The most widely studied activities of the PLs include the regulation of mammary gland secretory differentiation, ovarian steroidogenesis, the stimulation of fetal growth, the regulation of intermediary metabolism, immunoregulation and osmoregulation. Recent studies indicate that placental PLs, specifically rPL-I, are involved in the regulation of the characteristic PRL surges in the pregnant rat (Tomogane et. al., 1992).

A. Regulation of mammary gland secretory differentiation

During pregnancy, the mammary gland undergoes considerable growth and differentiation in preparation for lactation (Thordarson and Talamantes, 1987). Prolactin and hormones with PRL-like activities participate with steroid hormones and other peptide hormones in the regulation of growth responses and the acquisition of cellular machinery for synthesizing milk proteins (Southard et. al., 1987). It has been postulated that PRL can bind to PRL receptors on mammary gland tissue to regulate the ductal and alveolar growth of mammary epithelium and to control differentiation of mammary gland by initiation and

maintenance of lactogenesis. It is also known that PRL can directly regulate the transcription of the β-casein gene in the lactating mammary gland (Thordarson and Talamantes, 1987). The mPL-I, mPL-II and bPL have all been shown to stimulate mammary gland and α-lactalbumin synthesis (Colosi et. al., 1982; Colosi et. al., 1987; Thordarson et. al., 1986; Byatt et. al., 1986) while hPL has been shown to stimulate mammary gland epithelial cell proliferation and milk production (Welsh and McManus, 1977).

Recent studies show that mPL-I and mPL-II both stimulate mammary gland epithelial production of IGF-I binding protein I (IGF-I BP-I) and α-lactalbumin (English et. al., 1991). IGF-I BP-I facilitates growth while α-lactalbumin promotes differentiation of mammary gland. The mPL-I is a more potent stimulator of the synthesis of a 29 kDa IGF binding protein than mPL-II, while mPL-II is a more potent stimulator of α-lactalbumin synthesis than mPL-I (English et. al., 1991).

B. Regulation of fetal growth and metabolism

Recent studies have shown that hPL and oPL are involved in regulating the metabolism and growth of the fetus. Human PL has been reported to stimulate amino acid uptake, incorporation of [^{3}H] thymidine and IGF-I production by myoblasts and fibroblasts of human fetuses from the first half of gestation (Hill et. al., 1986; Swenne et. al., 1987). In human hepatocytes, hPL stimulated IGF-I production and the incorporation of [^{3}H] thymidine (Strain et. al., 1987). In fetal sheep, oPL has been reported to stimulate glycogen synthesis by the fetal liver (Freemark and Handwerger, 1986). PRL and GH had little or no activity

in ovine fetal hepatocytes or human fetal myoblasts and fibroblasts (Freemark and Handwerger, 1986; Freemark et. al., 1992; Hill et. al., 1985), although GH regulates these functions in postnatal animals. These data suggest that hPL and oPL might play important roles in regulating fetal metabolism and growth that are fulfilled by GH after birth.

There have been several case reports of normal sized infants born to mothers with very low plasma hPL concentrations resulting from deletions of two of the three hPL genes (Nielsen et. al., 1979; Borody and Carlton, 1981; Sideri et. al., 1983). In such cases, it appears that other hormones or factors may compensate for the absence of hPL. Using highly specific monoclonal antibodies to hPL and hGH, Frankenne and co-workers reassayed the hormone content of the placenta of a woman with deletions of the hCS-A, hCS-B and hGH-V genes whose pregnancy was characterized by an apparent lack of immunoreactive hPL in maternal blood and placental tissue. The "abnormal" placenta was found to contain hPL-like immunoreactivity although polyclonal antisera had originally detected none (Frankenne et. al., 1988). It was postulated that the "abnormal" placenta may produce hPL-like molecules through the expression of genes that are not expressed under normal conditions, such as the hCS-L gene and/or an altered hGH-N gene (Frankenne et. al., 1987; Cooke et. al., 1988). It is possible that these PL-like proteins could assume functions of hPL in the mother and/or fetus, sustaining normal fetal growth and development during those pregnancies complicated by a deficiency or absence of normal hPL production.

Current evidence, therefore, suggests that PLs may play a pivotal role in pregnancy, acting through distinct PLs receptors to regulate and to coordinate growth and metabolism

in the mother and fetus. In midpregnancy, PLs may secrete preferentially into the fetal circulation, exerting somatotropic effects at a time when the rate of linear growth of the fetus is maximal. Subsequently, during the latter half of gestation, the metabolic effects of PLs in the mother and fetus may predominate, insuring the optimal supply of nutrients to the fetus and their utilization by fetal tissues (Freemark and Handwerger, 1989).

The mPL-I, mPLF and rPLP-A have also been identified in the blood of fetal mice and rats. In the mouse, mPL-II is present in the fetal circulation from day 14 to day 16. The binding sites have been identified in the fetal mouse liver on day 17 of gestation (Harigaya et. al., 1988). However, the biological function of mPL-II in the fetus has not been determined. The functions of mPLF and rPLP-A in the fetus are also not known, although it has been suggested that mPLF may be involved in regulating placental and/or fetal growth since high concentration of mPLF have been observed during periods of growth in mouse 3T3 cell line (Linzer and Nathans, 1984). There is also evidence that mPLF may be involved in the regulation of muscle cell proliferation (Linzer et. al., 1985; Wilder and Linzer, 1989). The rPLP-A has been localized to the nucleus of trophoblast cells (Campbell et. al., 1989; Deb and Soares, 1990), which suggests that the nuclear localization of rPLP-A may be part of an autocrine control loop regulating trophoblast cell growth and differentiation.

C. Regulation of maternal intermediary metabolism

A number of changes in maternal intermediary metabolism occur during pregnancy. These include: 1) an increase in the insulin response to a glucose load, 2) resistance to the

effects of insulin in lipid mobilization, 3) a decrease in the glucose tolerance, (4) an increase in lipid mobilization (Freinkel et. al., 1985). The net effect of these changes is sparing of maternal glucose for utilization by the fetus. The involvement of hPL in bringing about these adaptations was suggested shortly after the hormone was purified (Grumbach et. al., 1968) and many results have been presented over the past two decades for roles of hPL in regulating maternal intermediary metabolism (Ogren and Talamantes, 1988). Effects of oPL and mPL-II on maternal intermediary metabolism have also been reported. Ovine PL has been shown to increase the circulating concentrations of free fatty acids, glucose and amino acids turnover in fed ewes (Thordarson et. al., 1987). Mouse PL-II, however, does not stimulate lipolysis *in vitro* in adipose tissue from pregnant mouse during second half of pregnancy (Fielder and Talamantes, 1987).

D. Regulation of steroidogenesis

The role of PLs in regulating steroidogenesis has been examined in the mouse, rat, human and sheep. These investigations have focused on the effects of PLs on the production of progesterone, which is required for the maintenance of pregnancy. It is well established that in both rat and mouse, substances secreted by the conceptus are important in regulating progesterone production by the ovaries during the second half of pregnancy (Linkie and Niswender, 1973; Crister et. al., 1980) These substances have not been identified, but it has been suggested that the PLs may be responsible for at least some of this luteotropic activity (Glaser et. al., 1984). The decidual luteotropin, a 28 kDa protein which is synthesized and

secreted by rat decidual tissue, has been shown to bind to PRL receptors and has functions similar to PRL, including stimulation of steroidogenesis in the corpus luteum (Herz et. al., 1986). Whether these observations are indications that members of the PRL family participate in the hormonal adjustment of pregnancy remains to be determined.

E. Functions in the immune system

Significant adjustments in the immune system must occur during pregnancy in order to permit survival of genetically disparate embryonic and extraembryonic tissues in the reproductive tract of the mother. Pituitary PRL, PL-I and PL-II each have prominent stimulatory effects on the proliferation of Nb2 rat lymphoma cell line (Tanaka et. al., 1980; Robertson et. al., 1982; Colosi et. al., 1987). Several lines of evidence indicate that the pituitary hormone PRL may be an important immunoregulatory hormone. *In vitro* studies in rats showed that both hypophysectomy and inhibition of PRL release with the dopaminergic agonist bromocriptine inhibit the development of delayed cutaneous hypersensitivity and antibody formation to sheep red blood cells (Berczi et. al., 1981; Nagy et. al., 1983). Treatment with exogenous PRL reverses these immunosuppressive effects (Berczi et. al., 1981). Recent studies showed that human lymphoid cells are able to synthesize and secrete human PRL and to express PRL receptors (Berczi et. al., 1991). The presence of the receptor for PRL and production of PRL by lymphocytes suggest a possible autocrine or paracrine effect of PRL in immune cell function. Pituitary PRL has also been shown to influence the behaviour of normal lymphocytes including stimulating lymphocyte proliferation (Hartmann

et. al., 1989; Berczi et. al., 1991), modulating IL-2 stimulated lymphocyte proliferation (Mukherjee et. al., 1990; Clevenger et. al., 1990) and regulating the transcription of specific lymphocyte genes (Yu Lee et. al., 1990).

F. Regulation of amniotic fluid and fetal hydromineral balance

In humans, decidual PRL is the major source of PRL in amniotic fluid (McCoshen and Barc, 1985). Human decidual PRL has been postulated to be involved in the regulation of water and ion transfer through extraembryonic membranes (Mulder, 1989). Human amnion and trophoblast cells have been shown to possess receptors for pituitary PRL (Ormandy et. al., 1990; Herrington et. al., 1980), which suggests that PRL receptors may mediate the action of PRL on extraembryonic tissues, or act as a means of transporting PRL into other extraembryonic or embryonic compartments.

Effects of PRL on water and mineral transport by fetal tissues have also been reported. Ovine PRL did not affect the permeability of fetal rhesus monkey skin to water, but the presence of PRL receptors in the fetal rhesus monkey lung has been reported (Jasimovich et. al., 1977). This is of interest because the fetal lung appears to be an important area of fluid exchange between the fetus and amniotic fluid. Human PL, mPL-II and mPLF are also present in amniotic fluid where their gestational profiles are similar to those in maternal blood (Grumbach et. al., 1968; Nielsen et. al., 1979; Colosi et. al., 1987; Linzer et. al., 1985). The function of these proteins in amniotic fluid is not known, although it was suggested that mPLF could act as an autocrine and/or paracrine growth factor for the fetus and the placenta

(Lee and Nathans, 1988).

G. Role of placental PRL family members in the control of the PRL surges

The pattern of PRL secretion in pregnant rats is characterized by two daily surges, which include a nocturnal and a diurnal surge, during the first half of pregnancy (Smith and Neill, 1976). These surges abruptly terminate at midpregnancy and PRL secretion remains low until shortly before parturition (Bridges and Goldman, 1975; Grattan and Averill, 1990). Termination of rPRL surges in pregnant rats occurs at the same time that the level of circulating rPL-I increases, and many reports suggest that PL-I may be responsible for termination of these surges (Tonkowicz and Voogt, 1983a; Tonkowicz and Voogt, 1983b; Voogt et. al., 1985; Voogt and de Greef, 1989). Recent studies using the rat choriocarcinoma Rcho-1 cell line which secretes rPL-I *in vivo* and *in vitro*, have supported this conclusion (Tomogane et. al., 1992). Injection of Rcho-1 cells under the kidney capsule of either pregnant or cycling rats which were ovariectomized one day before serotonin (an essential component in the generation of PRL surges) injection completely inhibited the serotonergic neuronal stimulus-induced PRL release. This result suggested that rPL-I may exert its effects by blocking the action of serotonin on dopamine neurons, leading to dopamine release and therefore inhibiting PRL secretion (Tomogane et. al., 1992). During the latter half of pregnancy, rPL-I declines and secretion of rPL-II and other PRL-related proteins increases. Since Rcho-1 cells in prolonged culture also secreted rPL-II, rPLP-A, rPLP-C and rPL-Iv (Faria and Soares, 1991), it is possible that during the latter half of pregnancy rPL-II and

other PRL-related proteins may take over the function of rPL-I in inhibiting PRL secretion. Several studies have suggested that progesterone is inhibitory to PRL secretion during late pregnancy as well (Grattan and Averill, 1990). Progesterone treatment on day 20 rats blocked the nocturnal PRL surge which normally occurs on the morning of day 21 (Grattan and Averill, 1990); the mechanism is not known.

In summary, although the biological functions of PRL and PRL-related proteins are not well understood, they appear to have multiple biological actions which include regulation of mammary gland secretory differentiation, of maternal intermediary metabolism and steriodogenesis, osmoregulation and immunoregulation as well as function as autocrine or paracrine growth factors for fetus and placenta.

VII. The rat prolactin-like protein A (rPLP-A)

Rat prolactin-like protein A (rPLP-A) was first discovered during the cDNA cloning of rPL-II from a day 18 rat placenta cDNA library and named rPLP-A because of its sequence similarity with PRL (Duckworth et. al., 1986b). Similar to other PRL-related proteins, rPLP-A is structurally more closely related to PRL than to GH. It is almost equally related to hPRL and rPRL (45% and 43%, respectively) whereas it is only 29% related to rGH, hGH and hPL. The mRNA corresponding to rPLP-A is 1 kilobase in length and first appears at day 14 of gestation, reaches a maximum at day 18 and remains high until term. The expression of rPLP-A is placenta specific; it is undetectable in other tissues examined

(Duckworth et. al., 1986b). *In situ* hybridization and immunocytochemistry experiments have shown that rPLP-A is localized in the trophoblast giant cells and spongiotrophoblast cells of the basal zone (Duckworth et. al., 1986b; Campbell et. al., 1989).

The amino acids sequence deduced from the nucleotide sequence of the rPLP-A cDNA suggested that rPLP-A is a secreted glycoprotein of 196 amino acids (predicted molecular weight is 25 kDa) with two potential N-linked glycosylation signals (Duckworth et. al., 1986b). Antibodies to three regions of the rPLP-A sequence (peptide #101-114, #129-145 and #152-164) were developed and used as specific immunological probes to characterize the rPLP-A proteins (Deb et. al., 1989). It was found that rPLP-A exists in placenta as two major molecular weight forms of 29 kDa and 33 kDa as well as two weak immunoreacting species of 26 kDa and 31 kDa. The 29 kDa and 33 kDa rPLP-A proteins were found to be glycoproteins of the 25 kDa precursor as determined by: 1) their high affinity for the lectin concanavalin-A; 2) their susceptibility to N-Glycanase digestion (treatment of placental cytosol preparation with N-Glycanase prior to immunoblotting resulting in the identification of only a 25 kDa protein instead of the 29 kDa and 33 kDa protein species); and 3) the inhibition of *de novo* glycosylation by tunicamycin (tunicamycin shifts the size of rPLP-A synthesized by placental explants from 29 kDa and 33 kDa to 25 kDa). The rPLP-A proteins can be detected in maternal serum and placental explant medium from day 13 to term as well as from day 19 fetal serum, but not in nonpregnant female and male rat sera (Campbell et. al., 1989; Deb et. al., 1989). Immunocytochemistry studies revealed that rPLP-A was localized in the nucleus as well as cytoplasm of giant cells and spongiotrophoblast cells in

the junctional zone. The nuclear localization of rPLP-A is not understood, but it was suggested that rPLP-A may influence processes in the nucleus (Campbell et. al., 1989) or act as an autocrine growth factor in regulating trophoblast cell growth and/or differentiation (Deb et. al., 1989).

During purification of rPLP-A from placental explants, Soares and co-workers found that rPLP-A is not a single monomeric secretory protein. The rPLP-A isolated from placental cytosol or from serum of pregnant rats predominantly exists as disulfide-linked high molecular weight complexes (62 kDa, 124 kDa and 132 kDa), which show weak but specific reactivities with rPLP-A antipeptide antisera (Deb and Soares, 1990). Denaturation and chemical reduction of high molecular weight complexes resulted in the generation of immunoreactive rPLP-A monomers (29 kDa and 33 kDa). The nature of the disulfide-linked complexes is unknown. The complexes may represent homooligomers or heterooligomers of rPLP-A binding with other serum proteins (Deb and Soares, 1990).

Both rPLP-A and hamster placental lactogen II (HaPL-II) circulate primarily as high molecular weight disulfide-bonded complexes (Deb and Soares, 1990; Southard and Talamantes, 1989). It seems likely that the unusual disulfide-bonding behaviour might be a reflection of the unique structural features of cysteine residues in rPLP-A and HaPL-II. Four cysteine residues in rPLP-A and hamster PL-II (HaPL-II) correspond to those of mPL-II and rPL-II (at positions 51, 166, 183 and 191) and are conserved in essentially identical locations in all known members of the PRL family. However, HaPL-II contains a pair of cysteine residues not present in the other members of the PRL family, which occur at residue 21 and

42. Like HaPL-II, mammalian PRLs have an additional pair of cysteine residues. The position of these cysteine residues in PRLs are highly conserved with both occurring within the first 11 amino acid residues of the mature proteins. Currently, complete amino acid sequences are available for most of the PRL-related proteins, none of these proteins contains cysteine residues corresponding to those at positions 21 and 42 in HaPL-II. This unique pair of cysteine residues may be responsible for the extreme tendency of HaPL-II to form disulfide-bonded complexes when compared with other members of the PRL family (Southard et. al., 1989). In the case of rPLP-A, there are five cysteine residues present in the rPLP-A structure (Duckworth et. al., 1986b), possibly permitting the formation of disulfide bonds with proteins possessing free cysteine residue. Given the similar location of the additional cysteine residue in rPLP-A (Cys^{101}) and mPRP (Cys^{119}), it is possible that mPRP also circulates as a disulfide-bonded complex, although this has not been examined. The nature of disulfide-linked HaPL-II and rPLP-A is not clear; they may represent a repository for biologically active monomers.

Purification of rPLP-A from placental explant and maternal serum has proved unsuccessful because of the formation of large molecular weight complexes and the contamination of other PRL-related proteins. Soares and co-workers have attempted to purify rPLP-A using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroelution. Their purified rPLP-A protein from placental explants contained contamination by several other PRL-related proteins, which later led to the identification of rPLP-C and rPL-Iv (Deb et. al., 1991b; Deb et. al., 1991c; Deb et. al., 1991a). The rPLP-A monomers have been reported to have lactogenic activity (Deb and Soares, 1990); however,

it was later proved to be due to contamination by rPL-Iv (Deb et. al., 1991a). The rPLP-A monomers purified from SDS-PAGE and electroelution (these processes could denature the protein) did not stimulate Nb2 cell proliferation. Soares and co-workers have shown evidence that rPLP-A circulates in the maternal and fetal sera as high molecular weight complexes (Deb and Soares, 1990) but to date both the function and gestational profile of rPLP-A are not known.

The *cis*- and *trans*-acting factors that are involved in the developmental expression of rPLP-A remain mostly unknown. Recent studies showed that a 975 bp fragment of the 5'-flanking sequence is sufficient to specify placental expression of the rPLP-A gene using Rcho rat Rcho choriocarcinoma cells (Vuille et al., 1993). The Rcho cells provide not only a transfectable cell system for the identification of the *cis*-acting sequences responsible for placental specific expression of these genes, but should also prove to be a rich source of the *trans*-acting protein factors which bind to these sequences.

VIII. Research objectives

Due to the presence of multiple members of PRL-related proteins during pregnancy, purification of PRL-related proteins has proved to be a arduous process, yielding only small amounts of proteins with low purity (Robertson et. al., 1982; Colosi et. al., 1987), which contained contamination by the other PRL-related proteins (Deb et. al., 1991b; Deb et. al., 1991c; Deb et. al., 1991a). In order to further characterize rPLP-A and study its regulation

in vivo, it is very important to obtain sufficient quantities of the purified rPLP-A proteins; the purified protein can then be used to develop a sensitive radioimmunoassay (RIA) to determine the gestational profile of rPLP-A in maternal and fetal circulation, to study the regulation of rPLP-A and look for target tissues. The following investigations have been performed to that end:

1. The transfection and amplification of a rPLP-A expression vector and the characterization of recombinant rPLP-A
2. The purification of recombinant rPLP-A
3. The development of an RIA and the study of the gestational profile of rPLP-A
4. The study of the regulation of rPLP-A during pregnancy

MATERIALS AND METHODS

I. Transfection and amplification of rPLP-A cDNA

1). Cell culture

DG44, a dihydrofolate reductase (DHFR)-deficient Chinese Hamster Ovary (CHO) cell (Chasin et. al., 1986) (kindly provided by Dr. L. Chasin, Columbia University, New York, N.Y.), was maintained in a 1:1 mixture of Dulbecco's modified eagle's medium (MEM); (GIBCO/BRL) and MCDB 302 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 µg/ml), 0.1 mM L-proline, 10 µg/ml hypoxanthine and 10 µg/ml thymidine in an incubator at 37°C in an atmospherer of 95% air, 5% CO_2 incubator.

2). Transfection and amplification of the rPLP-A expression vector

The full-length rPLP-A cDNA sequence was inserted into the pMSXND expression vector (Linzer et. al., 1986) (kindly provided by Dr. D. Linzer, Northwestern University, Evansville, Ill.). This vector contains a unique Xho I cloning site for the gene of interest under the control of a mouse metallothionein promoter, as well as a mouse dihydrofolate reductase gene under the control of a SV40 promoter. The latter gene allows for the amplification of the transfected gene when cells are grown in increasing concentrations of

methotrexate. Ten micrograms of the supercoiled expression construct were transfected into $1x10^6$ cells using Lipofectin™ Reagent (GIBCO/BRL) (Maurer and Notides, 1987). Transfected cells were selected for growth in medium containing 500 µg/ml G418 (1 mg/ml geneticin). G418-resistant colonies were cloned by use of a cloning cylinder and then selected by stepwise increases in methotrexate concentration, ranging from 0.08-200 µM. Individual colonies were cloned and assayed for rPLP-A expression by protein-labelling and immunoprecipitation of [^{35}S]-methionine-labelled conditioned medium using rPLP-A antipeptide antiserum generated against peptide #129-145.

3). SDS polyacrylamide gel electrophoresis and Immunoprecipitation

One-dimensional SDS-PAGE was performed according to the procedure of Laemmli (Laemmli, 1970). The individual clones of rPLP-A transfected CHO cells were incubated in 6 wells plates (Falcon Plastic) with 2 ml of methionine-free Dulbecco's MEM (GIBCO) for 18 hr and then labelled in the same medium containing 100 µCi/ml [^{35}S]-methionine for 8 hr. In each sample, the conditioned medium was collected and a volume containing 100,000 cpm of TCA precipitated [^{35}S]-methionine labelled proteins was incubated overnight at 4°C with 10 µl of preimmune rabbit serum in 200 µl of immunoprecipitation buffer (10 mM Tris HCl, pH 7.8, containing 150 mM NaCl, 10 mM L-methionine, 0.5% Nonidet P-40 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS and 2 mM PMSF). After incubation, the non-specific antigen-antibody complex was removed by incubating with 100 µl of swollen Protein A Sepharose-4B (Pharmacia). The supernatant was then incubated overnight with 10 µl of rPLP-

A antipeptide antiserum and the antigen-antibody complex were precipitated by Protein-A Sepharose-4B, washed 2 times with immunoprecipitation buffer containing 2.5 M KCl and once without KCl. After careful removal of excess fluid, the pellet was resuspended in 30 µl loading buffer (0.625 M TrisCl, pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol) and heated at 100°C for 5 min. Samples were then centrifuged and supernatants were recovered and electrophoretically separated on 12.5% SDS-polyacrylamide gels. The gels were dried and exposed to Kodak X-Omat AR X-ray film and developed. Cultures showing the largest amount of synthesis were selected for further amplification.

II. Characterization of recombinant rPLP-A

1). Comparison of recombinant rPLP-A with native rPLP-A:

The rPLP-A transfected CHO cells that were selected as the highest producers were grown to confluence with medium containing 10% dialysed fetal calf serum (which lack hypoxanthine), antibiotics and 200 µM methotrexate in 100 mm dish (Corning), washed with phosphate-buffered saline (PBS) and changed to medium which contained 0.1 mM L-proline and antibiotics, without serum and methotrexate and incubated for 24 hr at 37°C in 5% CO_2. The conditioned medium was then collected by centrifugation and concentrated by Centricon 10 (Amicon).

The placentas were dissected from rats on day 18 of pregnancy (day 1 is the day spermatozoa are detected in the vaginal smear). The placentas were immediately cut into

approximately 1 mm^2 pieces and incubated with serum-free medium containing antibiotics for 24 hr at 37°C in 5% CO_2. The conditioned medium from placental explants were then collected by centrifugation and concentrated by Centricon 10 (Amicon).

Equal amounts of total protein from cells and placental explants (determined by Bio-Rad Protein Assay) were fractionated by 12.5% SDS-PAGE, electrophoretically transferred and immunoblotted with rPLP-A antipeptide antiserum (described as below).

2). Effects of tunicamycin treatment on *de novo* rPLP-A synthesis from rPLP-A transfected CHO cells

The rPLP-A transfected CHO cells were preincubated for 1 hr in 100 mm dishes (Corning) containing 2 ml of serum-free medium with penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37°C in a 95% air, 5% CO_2 incubator. Following preincubation, the medium was replaced with 2 ml of fresh medium in the presence or absence of tunicamycin (10 µg/ml). The conditioned media were collected after 20 hr of incubation. Any cells present were removed by centrifugation. Equal amounts of protein from non-treated or tunicamycin-treated cultures were electrophoresed in a 12.5% SDS-polyacrylamide gel and stained with silver, or electrophoretically transferred to nitrocellulose (Towbin et. al., 1979).

3). Immunoblotting

After transfer, the nitrocellulose sheet was washed for 10 min with TBST (20 mM Tris HCl buffer, 150 mM NaCl, and 0.05% Tween 20, pH 8.0), and unoccupied sites were

blocked by incubation for 1 hr with 3% gelatin in TBST (blocking solution). The sheet was then incubated overnight with rPLP-A antipeptide antiserum diluted 1:1000 in blocking solution. After washing (three times, 10 min each) with TBST, the sheet was incubated for 1 hr with goat anti-rabbit γ-globulin alkaline phosphatase-conjugate (Promega) diluted 1:7500 in blocking solution. The nitrocellulose was further washed (three times, 10 min each) with TBST and developed using the Promega alkaline phosphatase color development reagents as described by the manufacturer. All incubation and washing steps were performed with gentle agitation on a rotary shaker.

4). Lectin Binding

In order to determine whether rPLP-A could bind to lectin to assistant in the purification of rPLP-A protein using lectin affinity chromatography, a lectin binding method was performed according to the procedure described by Holden and Rohringer (1985). Equal amounts of proteins from conditioned media of rPLP-A transfected CHO cells were loaded onto an SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose. The unoccupied sites were blocked by incubation for 1 hr with 3% gelatin in TBST. The nitrocellulose was then incubated with 25 μg/ml of biotin-labelled Concanavalin A (ConA) or Wheat Germ Lectin in TBST containing 1% gelatin, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ for 1 hr. After 3 washes of 10 min each, the sheet was incubated with avidin-labelled horseradish peroxidase (50 μg/ml) for 1 hr, followed by washes (3 times, 10 min each) and color development as described by the manufacturer.

5). Nb2 lymphoma cell bioassay

The Nb2 rat lymphoma cell line which was found to be PRL-dependent for growth, has been used to assay the bioactivity of lactogens (Tanaka et al., 1980). Nb2 lymphoma cells (11C) were used to estimate the lactogenic activity of recombinant rPLP-A conditioned medium. Nb2 cells were grown in Fisher's medium, containing 10% horse serum (HS), 10% fetal bovine serum (FBS), 10^{-4} M ß-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 µg/ml). Approximately 24 hr before their use in the bioassay, cells were transferred to Fisher's medium supplemented with 1% FBS, 10% HS, 0.1 mM ß-mercaptoethanol, antibiotics and incubated for 24 hr in order to slow down the rate at which the cells replicated. The cells were then collected by centrifugation (3 min at 300xg) and resuspended at a concentration of $1x10^5$ cells/ml in medium without FBS. Two ml aliquots (approximately $2x10^5$ cells) were distributed in 35-mm tissue culture dishes (Falcon Plastics). Samples to be assayed for growth promoting activity were added to these stationary cells in 100 µl PBS containing 0.1% bovine serum albumin (BSA). The cultures were incubated at 37°C in 5% CO_2 incubator for 72 hr, and the cell numbers were then determined using a Coulter counter (Coulter Electronics Inc.). All samples were assayed in duplicate. Growth in control cultures was essentially zero over the 3 days incubation period.

III. Purification of recombinant rPLP-A proteins

1). Collection and concentration of conditioned medium from transfected CHO cells:

The rPLP-A transfected CHO cells were grown to confluence on 150 mm dishes (Nunc), then fed with medium containing 0.1 mM L-proline and penicillin-streptomycin, but without serum and methotrexate. After 24 hr, the medium was removed and clarified by centrifugation, and fresh medium was added. Collection continued for 10 days, with daily harvesting of medium, yielding a total volume of 5 litres of conditioned medium. Two molar $ZnSO_4$ was added in to give a final concentration of 100 mM, and the solution was stirred for 15 min at 4°C. The precipitated proteins were collected by centrifugation at 13,000xg for 10 min, dissolved in 50 ml 0.5 M EDTA, pH8.0, containing 20 mM ß-mercaptoethanol, and dialysed extensively at 4°C against 50 mM HEPES, 100 mM NaCl, 10 mM EDTA and 20 mM ß-mercaptoethanol, 0.03% sodium azide, pH7.4 (Sephadex G-100 column buffer). The insoluble material was removed by centrifugation (10,000xg for 5 min).

2). Gel filtration chromatography

All protein purification procedures were performed at 4°C unless otherwise stated. The dialysed samples were applied to a 5x120 cm column of Sephadex G-100 (Fine, Pharmacia) equilibrated with the same buffer used for dialysis. The column was eluted at 4°C at a flow rate of 20 ml/hr. Fractions (12 ml) were collected and monitored by absorbance at 280 nm. To determine the location of the rPLP-A, equal amounts of protein were

electrophoresed on 12.5% SDS-PAGE. The gel was electrophoretically transferred and immunoblotted using rPLP-A antipeptide antiserum.

3). Concanavalin A (ConA) affinity chromatography

The rPLP-A antiserum positive fractions were pooled, concentrated by ultrafiltration by using Amicon membrane (UM 10), and dialysed extensively at 4°C against 20 mM Tris-HCl buffer, 150 mM NaCl, 10 mM EDTA, 2 mM ß-mercaptoethanol and 0.03% sodium azide, pH 7.2 (ConA column buffer). The sample was loaded onto a ConA Sepharose-4B (Pharmacia) column (5x7 cm) at room temperature. The column was washed with at least 4 bed volumes of buffer; the adsorbed proteins were eluted with buffer containing 0.5 M α-methyl-D-mannopyranoside and 0.5 M α-methyl-D-Glucopyranoside. The flow rate during chromatography was 10 ml/h and fractions were monitored by absorbance at 280 nm. Equal amounts of protein were resolved by SDS-PAGE, electrophoretically transferred and immunoblotted using rPLP-A antipeptide antiserum.

4). Protein measurement

Protein concentrations of fractions collected during purification were estimated by measuring the absorbance at 280 nm. The Bradford protein assay (Bio-Rad Laboratories) was employed for the protein determination of the final purified product, using BSA as a stardard (Bradford 1976).

IV. Development of RIA and measurement of rPLP-A during pregnancy

1). Iodination of rPLP-A peptide

An rPLP-A peptide (peptide #129-145) was iodinated by the Iodogen method (Salacinski et al., 1981). The reaction mixture consisted of 0.5 mCi carrier-free $Na^{125}I$, 5 Mg of rPLP-A peptide and 12.5 Ml of 0.1 M sodium phosphate, pH 7.0. After 8 min in the iodogen-coated tube, the reaction mixture was diluted with 500 Ml of 0.1% acetic acid, 0.1 M NaCl, 0.3% sodium azide, pH 3.5 (column buffer). The radiolabelled rPLP-A peptide was purified by gel filtration on a Sephadex G-25 (fine, Pharmacia) column (30x1.5cm) eluted with column buffer containing 0.1% BSA. The specific activity of the radiolabelled rPLP-A peptide, calculated from the elution profiles of the rasiolabelled peptide and free ^{125}I iodine on Sephadex G-25, was in the range of 60-80 μCi/μg peptide.

2). Development of a RIA:

A double antibody radioimmunoassay method was used for determining rPLP-A level. All hormone preparations, placental extracts, serum and rPLP-A tracer were diluted in 10 mM PBS, pH 7.4, containing 1% BSA (RIA buffer). Approximately 30,000-35,000 cpm [^{125}I] rPLP-A peptide in 100 μl of RIA buffer, 100 μl of rPLP-A peptide, protein standard, or assay samples, and 200 μl of RIA buffer were added to glass tubes (12x75cm). After 24 hr of incubation at 4°C, 100 μl of diluted rPLP-A antipeptide antiserum (1:3,000 dilution) was added to the mixture. After another 24 hr incubation at 4°C, 100 μl of sheep anti-rabbit γ-

globulin antiserum (1:23 dilution), and 100 µl of normal rabbit serum (1:100 dilution) were added. After a further 24 hr incubation at 4°C, the precipitates formed were centrifuged at 780xg for 30 min and the supernatants were decanted. The radioactivity in the precipitates was counted in a LKB γ-counter (model 800).

3). Animals:

Timed pregnant female Sprague-Dawley rats were bred in the Central Animal Care Facility, University of Manitoba. Animal maintenance and treatment were approved by the Animal Care Committee, Faculty of Medicine, University of Manitoba which conforms to the guidelines set by the Canadian Council on Animal Care. All animals were housed under temperature and light controlled conditions (lights on from 0500-1900), with free access to food and water. Day 1 was identified as the day when vaginal sperm were detected. Rats were decapitated between day 14 and term. Placentas were rapidly dissected, frozen on dry ice and stored at -70°C for placental extracts.

Amniotic fluid was collected by dissection of the uterus and removal of fetuses; the amniotic membranes were then ruptured to let the amniotic fluid drain off into glass tubes. A pool of amniotic fluid was collected. The amniotic fluids were chilled in ice and centrifuged within 2 hr and stored at -20°C until assay.

Blood samples were collected from tail veins of rats under light ether anaesthesia every other day beginning at day 12 of gestation, or as trunk blood after decapitation at day 21. Upon withdrawal, the blood was immediately chilled in ice, centrifuged at 4°C within 2

hr of collection and aliquots of serum were stored in stoppered glass vials at -20°C until assay.

4). Placental extracts:

Rat placental tissues from different days of gestation were homogenized in 0.1 M NH_4HCO_3 and 0.1 M NaCl, 0.5 mM PMSF, pH 9.3 at 4°C with a tissue to buffer ratio of 1:10 (wt/vol) in a Brinkmann polytron tissue homogenizer. Homogenates were further disrupted by sonication for 1 min before boiling at 100°C for 15 min, and then centrifugation at 15,000 g for 20 min. The pellets were discarded and the supernatants were stored at -20°C until assay. The total protein concentration in placental extracts was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories).

V. Regulation studies of rPLP-A during pregnancy:

1). Animals:

Timed pregnant rats were as described above.

2). Surgical procedures:

Ovariectomy and adrenalectomy in rats were performed under ether anaesthesia at day 16 of pregnancy. Care was taken to maintain clean operating conditions and to keep the rats

warm and comfortable during the initial postoperative recovery period. Ovaries and adrenals were removed through a dorsolateral opening at the second and third lumbar vertebral level. Before removal, the ovaries were freed of the covering adipose tissue and their vessels were ligated with silk thread. Adrenals were removed intact in their capsules, together with the surrounding adipose tissue. The incision was closed with silk sutures and skin clips. Adrenalectomized rats were maintained on a 0.9% NaCl drinking solution. A group of sham-operated rats was included in the experiment.

3). Measurement of rPL-II and rPLP-A:

Radioimmunoassays were used for the determination of rPLP-A and rPL-II concentrations in different days of pregnant rat sera after ovariectomy, adrenalectomy or ovariectomy plus adrenalectomy performed at day 16 of gestation. A RIA for rPLP-A was described above. The rPL-II, purified by an antibody affinity column, was iodinated by the iodogen method. The [^{125}I]-labelled rPL-II was purified by gel filtration on a Sephadex G-100 (fine, Pharmacia) column (30x1.5 cm) eluted with PBS, pH7.4 containing 0.1% BSA. In the RIA for rPL-II, each tube contained 100 µl of standard or unknown sample, 100 µl of anti-rPL-II antibody at a dilution of 1:4,000, 200 µl of RIA buffer (PBS containing 1% BSA and 25 mM EDTA) and 100 µl of [^{125}I] rPL-II (30,000 cpm). After incubation for 24 hr at 4°C, normal rabbit serum (1:100) and sheep anti-rabbit γ-globulin antiserum (1:23) were added and 1 day later "bound" and "free" rPL-II were separated by centrifugation and radioactivity was counted.

When comparisons were made between treatment and sham-operated groups, all samples were assayed in the same RIA. The statistical difference between treatment means were tested by analysis of variance using Tukey's test. In all cases, $P < 0.05$ was considered significant.

VI. Production of rPLP-A/GST fusion protein

1). Cloning of a rPLP-A cDNA into the pGEX-2T vector and screening for a fusion protein:

The pGEX-2T vector (Smith and Johnson, 1988) was used in Escherichia coli (E. coli) to express rPLP-A as fusions with glutathione S-transferase (GST). The rPLP-A cDNA (missing the N-terminal 24 amino acids) was cut with EcoRV and EcoRI. The EcoRI site was blunt-ended using Klenow fragment of DNA polymerase, then cloned into the SmaI site of pGEX-2T vector and introduced into E. coli by transformation. A numbers of transformants were grown in LB medium containing 100 μg/ml of ampicillin in the presence of isopropy-B--D-thiogalactopyranoside (IPTG) in order to induce the expression of the fusion protein and were screened for the expression of fusion protein by electrophoresis of total bacterial lysate on a 10% SDS-polyacrylamide gel. A single subclone was selected from 24 colonies for protein purification.

2). Purification of rPLP-A/GST fusion protein:

The rPLP-A/GST fusion protein positive colony was inoculated into 100 ml of LB medium containing 100 μg/ml of ampicillin and grown for 16 hr at 37°C in a shaking incubator. This overnight culture was diluted 1:10 into 1 litre of fresh LB medium containing 100 μg/ml of ampicillin and grow for 1 hr at 37°C. IPTG was then added to 0.1 mM and incubation continued for an additional 5 hr. Purification of rPLP-A/GST fusion protein using glutathione affinity column proved unsuccessful because more than 90% of the fusion protein was insoluble. Therefore the rPLP-A/GST fusion protein was purified by SDS-PAGE and electorelution described as below. Cells were collected by centrifugation at 5,000xg for 10 min. The pellet was collected in 2 ml of SDS sample buffer, boiled at 100°C for 5 min and then fractionated by 10% SDS-PAGE. Slices (0.5 cm on each side) of the gel were cut and stained with 0.1% Coomassie blue R-250, destained and the fusion protein band was identified. The stained gel slices were aligned with the remaining gel and the unstained fusion protein band was cut out. The fusion protein was electroeluted with 50 mM NH_4HCO_3 and 0.1% SDS at 60 mA for 5 hr and electrodialyzed in a Model 422 Electroeluter against 50 mM NH_4HCO_3 containing 0.01% SDS at 60 mA for 24 hr with 3 buffer changes using. The concentration of purified rPLP-A/GST fusion protein was determined by Bio-Rad Protein Assay (Bio-Rad Laboratory).

RESULTS

A. Characterization of the recombinant rPLP-A

The full-length rPLP-A cDNA coding sequence including the signal peptide was inserted into the eukaryotic expression vector (pMSXND) downstream from the mouse metallothionein I promoter; this vector (Linzer et. al., 1986) also contains the mouse dihydrofolate reductase cDNA and neomycin resistance gene. The pMSXND/rPLP-A construct was then introduced into DG44, a Chinese Hamster Ovary (CHO) cell line which is dihydrofolate reductase deficient, and stable G418-resistant transformants were obtained. The G418-resistant clones were grown in increasing concentrations (0.8-200 μM) of methotrexate to select for dihydrofolate reductase gene amplification, and consequently, coamplification of the linked rPLP-A cDNA. Several clones which secreted high levels of rPLP-A were obtained and analyzed for rPLP-A expression by immunoprecipitation of the ^{35}[S]-methionine labelled secreted proteins with rPLP-A antipeptide antiserum (Fig. 3). Levels of rPLP-A expression in these clones varied considerably, and the secreted protein had a greater degree of size heterogeneity (26 kDa, 29 kDa, 31 kDa and 33 kDa) than rPLP-A in the placental extract (mainly 29 kDa and 33 kDa) (Fig. 4) although another studies showed that 26 kDa and 31 kDa rPLP-A protein can also be detected as minor secreted proteins from placental explants (Deb et al., 1989). The clone expressing the highest level of rPLP-A was

chosen as the source of the rPLP-A protein for the purification.

The amino acids sequence deduced from the nucleotide sequence of the rPLP-A cDNA suggests that rPLP-A is a secreted protein of 196 amino acids with two potential glycosylation signals. Two experiments were performed to evaluate the glycoprotein nature of the recombinant rPLP-A protein. 1). Treatment of rPLP-A expressing cells with tunicamycin prior to immunoblotting resulted in the production of only a 25 kDa protein (as indicated by arrow) rather than four protein bands (26, 29, 31, 33 kDa) as seen in the control (Fig. 5). The size of the protein in the presence of tunicamycin (25 kDa) is the same as the molecular weight predicted by the cDNA deduced amino acid sequence. It is also the same size as the rPLP-A produced by placental explants after tunicamycin treatment (Deb et. al., 1989). 2). It was also determined that the recombinant rPLP-A proteins have a higher affinity for Concanavalin A (ConA) than Wheat Germ Lectin when using a protein blotting method (Fig. 6).

B. Purification of the recombinant rPLP-A

Large scale cultures of the highest rPLP-A expressing clone were grown to confluency in serum containing medium, then switched to serum-free medium for collection. The conditioned medium was harvested and protein was precipitated with zinc sulfate. The protein was solubilized, dialysed, and fractionated by gel filtration using a Sephadex G-100 column (Fig. 7). Column fractions were assayed by SDS-PAGE followed by silver staining and

immunoblotting with rPLP-A antipeptide antiserum (Fig. 8). The fractions containing rPLP-A (fractions 65-80) were pooled, dialysed against ConA column buffer and chromatographed on a ConA Sepharose column (Fig. 9), with the yield of 2.1 mg of rPLP-A protein from 2.5 liters of conditioned media (Fig. 10).

C. Detection of rPLP-A in maternal and fetal circulation

A radioimmunoassay specific for rPLP-A was developed using ^{125}[I]-labelled rPLP-A peptide (129-145). Both the rPLP-A peptide and purified recombinant rPLP-A protein showed parallel displacement curves when compared with molarity calculated from mature rPLP-A protein and peptide #129-145 (Fig. 11), but the conditioned medium from DG44 cells did not. The concentration of the purified recombinant rPLP-A protein was estimated by the method of Bradford, using BSA as the standard. Displacement curves for dilutions of serum and placental extracts from pregnant rats were parallel to the rPLP-A peptide standard curve as well as to the purified recombinant rPLP-A protein (Fig. 12). The specificity of the assay was evaluated by examining the ability of rPRL, rGH, mPL-I, mPL-II, rFSH and rLH to displace ^{125}I-labelled rPLP-A peptide in the RIA. Sera from male and nonpregnant female rat as well as high concentrations (1 mg/ml) of rPRL, rGH, rPL-I, rPL-II, rFSH and rLH did not displace ^{125}[I]-rPLP-A peptide. The intraassay (three pregnant rat sera assayed 10 times in the same assay) and interassay (six pregnant rat sera assayed in five consecutive assays) coefficients of variation were 10.5% and 8.2%, respectively.

The rPLP-A concentration in the maternal and amniotic fluid was measured according to the dilution curve of the purified recombinant rPLP-A protein. The gestational profile of the rPLP-A concentration in the maternal serum as well as rPLP-A concentration in day 20 amniotic fluid is shown in Fig. 13. The rPLP-A was not detectable in serum samples from day 12 of pregnancy, its concentration increased gradually by day 14 (127 ng/ml) and then dramatically on day 20 (220 ng/ml). The concentration of the rPLP-A in day 20 amniotic fluid was relatively higher (317 ng/ml) compared with the serum level at day 20. It was confirmed by immunoblotting that day 20 amniotic fluid contained high levels of rPLP-A protein which is identical to the rPLP-A from placental extracts (Fig. 14).

D. Effects of ovariectomy and adrenalectomy on serum rPLP-A and rPL-II concentration during pregnancy

There is no information regarding the regulation of rPLP-A protein to date. However, previous studies have suggested that a number of factors are involved in the regulation of rPL-II which include factors from the ovary, adrenal, fetus and maternal pituitary. To determine the potential effects of the ovary and adrenal on rPLP-A and rPL-II secretion, ovaries and adrenals were removed from pregnant rats at day 16 of gestation. Ovariectomy of the rats led to increases in serum levels of rPLP-A and rPL-II which were significantly different on day 18, 19, 20 and 21 for rPLP-A and day 20 and 21 for rPL-II when compared with that of sham operated rats (Fig. 15 and 16). When adrenalectomy was carried out on day

16 of gestation, there was a slight but insignificant increase in serum rPLP-A and rPL-II levels above those found in sham operated animals. However, if adrenalectomy was combined with ovariectomy, further increases in serum levels of rPLP-A and rPL-II were seen above that produced by ovariectomy alone, showing significant differences with the sham operated group from 1 day after operations (day 17) until day 21. The results for rPL-II concentration after ovariectomy and/or adrenalectomy were very similiar to an earlier report (Robertson et. al., 1984a).

E. Characterization and purification of rPLP-A/GST fusion protein

In our laboratory, we have only one rPLP-A antipeptide antiserum which works well in immunoprecipitation, immunoblotting as well as in the radioimmunoassay. However, the antipeptide antiserum could not be used in an antibody affinity column for the purification of rPLP-A protein from either placental explants or rPLP-A cDNA transfected DG44 cells M.L. Duckworth, personal communication). Large amounts of purified rPLP-A protein are needed for the purpose of raising antiserum against rPLP-A protein. Attempts to raise antibody using purified recombinant rPLP-A protein were unsuccessful because the purification of a large amounts of the recombinant rPLP-A was tedious and costly. Therefore, a rPLP-A/GST fusion protein was produced to generate more pure rPLP-A protein for the purpose of raising antiserum. The rPLP-A cDNA missing 24 amino acids from the amino-terminus was joined in frame downstream from the translation initiation site of the

glutathione-S-transferase (GST) gene in E. coli plasmid pGEX2T which would allow the purification of the protein on a glutathione agarose column. SDS-PAGE of the total bacterial lysate was performed to screen for the expression of the rPLP-A/GST fusion protein and Only one out of 24 colonies contained rPLP-A/GST fusion protein (Fig. 17). More than 90% of the rPLP-A/GST fusion protein was insoluble in the buffer which made it difficult to purify the fusion protein by a glutathione agarose column. Therefore the rPLP-A/GST fusion protein was purified by cutting the fusion protein band (43 kDa) from Coomassie blue R-250 stained gel, electroeluting and electrodialysing with NH_4HCO_3 and SDS. The purified fusion protein (5 μg and 10 μg respectively) was separated by 10% SDS-PAGE and found to be purified to homogeneity as determined by Coomassie staining (Fig. 18). The purified rPLP-A/GST protein will be used for the generation of rPLP-A antiserum.

DISCUSSION

The initial attempts to purify rPLP-A protein from placental explant by Soares and co-workers, proved unsuccessful due to the presence of multiple members of the PRL-related proteins family during pregnancy. Preparations of rPLP-A from placental explants contained various contaminant PRL-related proteins which included rPL-Iv and rPLP-C (Deb et. al., 1991a; Deb et. al., 1991b). They also form large molecular weight rPLP-A complexes (Deb and Soares, 1990). In order to further characterize rPLP-A protein and study its regulation during pregnancy, it is necessary to obtain sufficient quantities of the purified rPLP-A proteins to develop a sensitive radioimmunoassay (RIA) to determine the gestational profile of rPLP-A in maternal and fetal circulation as well as to study the regulation of rPLP-A synthesis and secretion. To achieve these aims, a mammalian expression vector, pMSXND, was chosen. It was first developed by Lee and Nathans (Lee and Nathans, 1987) for the expression of the PRL-related mouse proliferin (mPLF) to express and purify recombinant rPLP-A. The vector has been used successfully for the production of mouse proliferin related protein (mPRP) (Colosi et. al., 1988b) and mouse PL-I (Colosi et. al., 1988a). Placental rPLP-A is known to be secreted as a glycosylated protein. One advantage of expression of rPLP-A in an mammalian cell system over the bacterial system is that glycosylation of protein can occur. Howevwe, the pattern of glycosylation of proteins expressed by mammalian cells such as the CHO cells we used and by placenta may not be the same (Lee and Nathans, 1987;

Colosi et. al., 1988b). As shown in Fig. 4 and 5, the recombinant rPLP-A proteins secreted by DG44 cells are a mixture of four different glycosylated species (26 kDa, 29 kDa, 31 kDa and 33 kDa) expressed at almost equal quantity, rather than rPLP-A proteins produced by placental explant which contain two major species (29 kDa and 33 kDa) and two minor species (26 kDa and 31 kDa). The difference in molecular weight patterns appears to be due to differences in the structures of N-linked oligosaccharides of recombinant and native rPLP-A proteins since tunicamycin treatment of rPLP-A transfected DG44 cells produce only the 25 kDa rPLP-A precursor which was indicated by arrow (Figure 5), as is the case for placental rPLP-A (Deb et. al., 1989). Despite the minor structural differences between recombinant and native rPLP-A, the proteins showed highly similar behaviour in their immunological characteristics.

Our initial attempts to purify recombinant rPLP-A using a Sephadex G-100 column, proved unsuccessful. The recombinant rPLP-A proteins, similar to placental rPLP-A, formed large molecular weight complexes since most of the rPLP-A immunoareactivity eluted in the void volume. Before attempting further purification, investigations were performed to determine whether the apparent heterogeneity of the recombinant rPLP-A might be due to the presence of disulfide-linked aggregates. The effect of ß-mercaptoethanol on the rPLP-A protein was investigated after we studied the procedures for the purification of hamster PL-II (HaPL-II) which also formed large molecular weight complexes during protein purification (Southard et. al., 1987). In the absence of ß-mercaptoethanol, the recombinant rPLP-A contained a major peak of rPLP-A immunoreactivity fractions which eluted in the void

volume (data not shown). Most of the rPLP-A immunoreactivity fractions after the void volume peak eluted in a position indicating a molecular weight of greater than 30,000. In the presence of 20 μM ß-mercaptoethanol, almost all of the rPLP-A immunoreactivity was present in a single major peak with an apparent molecular weight of 30,000. Some residual staining remained at higher molecular weights. These findings suggested that the inclusion of ß-mercaptoethanol in the buffers used for purification of rPLP-A might eliminate some or all of the heterogeneity that had made purification of this hormone previously very difficult.

Both rPLP-A and hamester PL-II (HaPL-II) circulate primarily as high molecular weight disulfide-bonded complexes (Deb and Soares, 1990; Southard et. al., 1987). Similar complexes may also form with other PRL-like proteins which contain only conserved cysteines (mPL-II and human PL), but may be to a less extent. Extensive complex formation probably requires the presence of one or more additional, nonconserved cysteine residues. The availability of a free sulfhydryl group is not sufficient for complex formation, since mPL-I which contains 9 cysteines circulates exclusively as monomers (Ogren et. al., 1989); on the other hand, purified HaPL-II, which does not contain a free sulfhydryl group, readily forms disulfide-bonded complexes, indicating that complex formation does not require a free sulfhydryl on the PRL-like proteins. Given the very similar location of the additional cysteine residue in rPLP-A (Cys^{96}) and mPRP (Cys^{101}) it is possible that mPRP also circulates as a disulfide-bonded complex, although this has not been examined. It is not known also whether the circulating complex of rPLP-A is similar to that of HaPL-II, which contains a pair of additional cysteines at positions 21 and 42.

During the preparation of this thesis, Soares and his co-workers published their purification of recombinant rPLP-A protein and identification of serum binding proteins (Deb et. al., 1993). They could not purify rPLP-A proteins to homogeneity; instead, they had to separate the rPLP-A monomers by SDS-PAGE and electroelution. Both procedures denature the proteins and separate the disulfide-bonded complexes. They also showed data indicating that rPLP-A binding proteins were present in serum from male and female rats. The concentration of the rPLP-A binding protein appeared to increase as gestation progressed. The rPLP-A binding proteins are not related to proteins previously implicated in binding members of the PRL/GH family, α_2-macroglobulin (Southard and Talamantes, 1989), mannose 6-phosphate receptor (Lee and Nathans, 1988), growth hormone-binding proteins (Sadeghi et. al., 1990). In addition, rPLP-A binding proteins are not restricted to pregnant rats. They are present in serum from both males and females, which might suggest a more fundamental role for rPLP-A or that they are non-specific binding proteins. Whether rPLP-A binding proteins function as receptors, as binding proteins to deliver rPLP-A to its appropriate target tissues, or represent a repository for biologically active rPLP-A monomers, is still not known.

Earlier studies showed that rPLP-A circulates in maternal serum (Deb et. al., 1989), but to date the level of the proteins have not been precisely measured. A double antibody immunoassay was therefore developed to study the gestational profile of maternal and fetal circulation and the regulation of rPLP-A during pregnancy. The data presented here clearly show that rPLP-A appears in the maternal serum by day 14 of pregnancy and reaches maximum levels at day 20. This profile corresponds well to that of rPLP-A mRNA level

detected during pregnancy (Duckworth et. al., 1986b). However to our surprise, rPLP-A protein was found in day 20 amniotic fluid at higher concentrations than in Day 20 maternal serum. This observation was confirmed by immunoblotting of amniotic fluid by rPLP-A antipeptide antiserum. A few members of the PRL/GH family, like hPL (Grumbach et. al., 1968; Nielsen et. al., 1979), mPL-II (Colosi et. al., 1987) and mPLF (Lee et. al., 1988) have also been detected in high concentrations in the amniotic fluid. The high concentration of rPLP-A protein in the amniotic fluid indicates that rPLP-A might function as an autocrine or paracrine growth factor for the development and differentiation of the fetus.

There is no information regarding the regulation of rPLP-A proteins to date. However, previous experiments indicated that the ovary, adrenal and pituitary factors are involved in the regulation of rPL-II. In order to study the regulation of rPLP-A under similar situations, ovariectomy and adrenalectomy were performed on day 16 pregnant rats to study the effects of endocrine ablation on rPLP-A levels during pregnancy. The results show that when adrenalectomy (Adx) was carried out on day 16 of gestation, there was a slight but not significantlly increase in serum rPLP-A and rPL-II levels above those found in sham operated rats ($p>0.05$, $n=3$). When ovariectomy was performed on day 16, serum rPLP-A and rPL-II levels were significantly higher on day 19 and 20 after operations ($p<0.05$, $n=3$). However, if adrenalectomy was combined with ovariectomy, an increase in serum rPLP-A and rPL-II was seen from day 17 until day 21 ($p<0.01$, $n=3$). The profound differences in serum rPLP-A and rPL-II levels seen in Adx/Ovx rats suggested that adrenal and ovarian steroids may play a role in the regulation of PLs. The previous experiments on regulation of rPL-II after

ovariectomy and adrenalectomy showed that neither progesterone, estrogen, nor progesterone plus estrogen were able to reduce serum levels of rPL-II in ovariectomized rats, which suggests that the increase in serum PLs levels after ovariectomy may be due to the removal of undetermined ovarian inhibitors acting on PL-II secretion (Robertson et. al., 1984a; Robertson et. al., 1984b). It is possible that the removal of the ovarian inhibitory factors is the mechanism for the regulation of rPLP-A secretion as well. However, since the loss of ovaries and adrenals has a profound effect on pregnancy (only 1 or 2 fetuses survived in the experiments), other regulatory mechanism may be involved as well.

SUMMARY AND FUTURE DIRECTIONS

The purpose of this research was the characterization and purification of recombinant rPLP-A protein, the development of a radioimmunoassay to study the gestational profile of rPLP-A in maternal and fetal circulation as well as the study of the regulation of the rPLP-A secretion.

The rPLP-A is a member of the PRL/GH gene family with some special features: 1) It contains 5 cysteine residues which allows rPLP-A to circulate in serum as large molecular weight complexes. The biological significance of the circulating high molecular weight rPLP-A proteins remains to be determined. 2) rPLP-A is present in both maternal and fetal circulation which might indicate that rPLP-A may function as an autocrine or paracrine growth factor for fetal and/or placental development. 3) Nuclear localization of rPLP-A, as reported by Deb and Soares (1990), may be part of an autocrine control loop regulating trophoblast cell growth and/or differentiation. Trophoblast cells have been shown to possess prolactin receptors (Boutin et. al., 1989) and polypeptide hormone and growth factors, including prolactin, have been localized to the nuclear compartment (Buckley et. al., 1988). The rPLP-A bound to its receptor in the nucleus may act similarly to nuclear bound prolactin which influence protein phosphorylation and ultimately gene expression (Buckley et. al., 1988). The validity of this autocrine hypothesis for rPLP-A action will have to await future experimentation.

As mentioned above, the rPLP-A protein is one of the most interesting members in the PRL/GH family. The further understanding of the function and regulation of rPLP-A could provides an insight into its role(s) during pregnancy. Tissue specific expression by Rcho-1 cell line, will faciliatate further investigations in placental hormone regulation as well as provide a model for placenta-specific gene expression (Vuille et al., 1993). It is also hoped that the identification of tissue and cell specific *cis* elements as well as *trans* acting proteins using transgenic mice may provide some clue as to its functional importance during pregnancy.

Adams, S.O., S.P. Nissley, S. Handwerger and M.M. Rechler (1983) Developmental patterns of insulin-like growth factor-I and -II synthesis and regulation in rat fibroblasts, Nature, 302, 150-153.

Ali, S., I. Pellegrini and P.A. Kelly (1991) A prolactin-dependent immune cell line (Nb2) expresses a mutant form of prolactin receptor, J. Biol. Chem., 266, 20110-20117.

Astwood, E.B. and R.O. Greep (1938) A corpus luteum stimulating substances in the rat placenta, Proc. Soc. Exp. Biol. Med., 38, 713-721.

Barlow, J.W., M.L.J. Voz, P.H. Eliard, M. Mathy-Hartert, P. DeNayer, I.V. Economidis, A. Belayew, J.A. Martial, G.G. Rousseau (1986) Thyroid hormone receptors bind to defined regions of the growth hormone and placental lactogen genes, Proc. Natl. Acad. Sci. USA, 83, 9021-9034.

Barnard, R., P.G. Bundesen, D.B. Rylatt and M.J. Waters (1985) Evidence from the use of monoclonal antibody probes for structural heterogeneity of the growth hormone receptor, Biochem. J., 231, 459-468.

Barrera Saldana, H.A., P.H. Seeburg and G.F. Saunders (1983) Two structurally different genes produce the same secreted human placental lactogen hormone, J. Biol. Chem., 258, 3787-3793.

Baumann, G., K.D. Amburn and T.A. Buchanan (1987) The effect of circulating growth hormone-binding protein on metabolic clearance, distribution, and degradation of human growth hormone, J. Clin. Endocrinol. Metab., 64, 657-660.

Baumbach, W.R., D.L. Horner and J.S. Logan (1989) The growth hormone-binding protein in rat serum is an alternatively spliced form of the rat growth hormone receptor, Genes Dev., 3, 1199-1205.

Bazan, J.F. (1989) A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain, Biochem. Biophys. Res. Commun., 164, 788-795.

Bazan, J.F. (1990) Structural design and molecular evolution of a cytokine receptor superfamily, Proc. Natl. Acad. Sci. U. S. A., 87, 6934-6938.

Ben Jonathan, N., L.A. Arbogast and J.F. Hyde (1989) Neuroendocrine [corrected] regulation of prolactin release [published erratum appears in Prog Neurobiol 1990;34(2):following 183], Prog. Neurobiol., 33, 399-447.

Berczi, I.E., Nagy, S.M. de Toledo, R.J. Matusik and H.G. Friesen (1991) Pituitary hormones regulate c-myc and DNA synthesis in lymphoid tissue, J. Immunol., 146, 2201-2206.

Berczi, I.E., Nagy, K. Kovacs and E. Horvath (1981) Regulation of humoral immunity in rats by pituitary hormones, Acta Endocrinol. Copenh., 98, 506-511.

Berle, P. (1974) Pattern of the human chorionic somatommamotropin (hCS) concentration ratio in maternal and amniotic fluid during normal pregnancy, Acta Endocrinologica, 76, 364-368.

Bewley, T.A. (1977) The chemistry of human chorionic somatomammotropin, Hormonal Proteins Peptides, 4, 61-65.

Bhaumick, B., E.P. Dawson and R.M. Bala (1987) The effects of insulin-like growth factor-I and insulin on placental lactogen production by human term placental explants, Biochem. Biophys. Res. Commun., 144, 674-682.

Borody, I.B. and M.A. Carlton (1981) Isolated defect in human placental lactogen synthesis in a normal pregnancy: case report, Br. J. Obstet. Gynaecol., 88, 447-451.

Boutin, J.M., C. Jolicoeur, H. Okamura, J. Gagnon, M. Edery, M. Shirota, D. Banville, I. Dusanter Fourt, J. Djiane and P.A. Kelly (1988) Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family, Cell, 53, 69-77.

Boutin, J.M., M. Edery, M. Shirota, C. Jolicoeur, L. Lesueur, S. Ali, D. Gould, J. Djiane and P.A. Kelly (1989) Identification of a cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells, Mol. Endocrinol., 3, 1455-1461.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem 72, 248-254.

Braverman, M.B., A. Bagni, D. de Ziegler, T. Den and E. Gurpide (1984) Isolation of prolactin-producing cells from first and second trimester decidua, J. Clin. Endocrinol. Metab., 58, 521-525.

Bridges, R.S. and R.B.D. Goldman (1975) Ovarian control of prolactin secretion during late pregnancy in the rat, Endocrinology, 97, 496-503.

Buckley, A.R., C.W. Putnam and D.H. Russell (1988) Prolactin as a mammalian mitogen and tumor promoter, Adv. Enzyme Regul., 27, 371-391.

Byatt, J.C., K. Shimomura, T.M. Duello and R.D. Bremel (1986) Isolation and

characterization of multiple forms of bovine placental lactogen from secretory granules of the fetal cotyledon, Endocrinology, 119, 1343-1350.

Campbell, W.J., S. Deb, S.C. Kwok, J.A. Joslin and M.J. Soares (1989) Differential expression of placental lactogen-II and prolactin-like protein-A in the rat chorioallantoic placenta, Endocrinology, 125, 1565-1574.

Cattini, P.A., N.L. Eberhardt (1987) Regulated expression of chimaeric genes containing the 5'-flanking regions of human growth hormone-related genes in transiently transfected rat anterior pituitary tumor cells, Nucleic Acids Res., 15, 1297-1305.

Chan, J.S.D., H.A. Robertson and H.G. Friesen (1978) Maternal and fetal concentration of ovine placental lactogen measured by radioimmunoassay, Endocrinology, 102, 1606-1613.

Chatelain, A., J.P. Dupuoy and M.P. Dubois (1979) Ontogenesis of cells producing polypeptide hormones (ACTH, MSH, LPH, GH, prolactin) in the fatal hypophysis of the rat: influence of the hypothalamus, Cell Tissue Res., 196, 409-453.

Chen, E.Y., Y.C. Liao, D.H. Smith, H.A. Barrera Saldana, R.E. Gelinas and P.H. Seeburg (1989) The human growth hormone locus: nucleotide sequence, biology, and evolution, Genomics, 4, 479-497.

Clevenger, C.V., D.H. Russell, P.M. Appasamy and M.B. Prystowsky (1990) Regulation of interleukin 2-driven T-lymphocyte proliferation by prolactin, Proc. Natl. Acad. Sci. U. S. A., 87, 6460-6464.

Colosi, P., G. Marr, J. Lopez, L. Haro, L. Ogren and F. Talamantes (1982) Isolation, purification and characterization of mouse placental lactogen, Proc. Natl. Acad. Sci. U. S. A., 79, 771-776.

Colosi, P., F. Talamantes and D.I. Linzer (1987) Molecular cloning and expression of mouse placental lactogen I complementary deoxyribonucleic acid, Mol. Endocrinol., 1, 767-776.

Colosi, P., L. Ogren, J.N. Southard, G. Thordarson, D.I. Linzer and F. Talamantes (1988a) Biological, immunological, and binding properties of recombinant mouse placental lactogen-I, Endocrinology, 123, 2662-2667.

Colosi, P., J.J. Swiergiel, E.L. Wilder, A. Oviedo and D.I. Linzer (1988b) Characterization of proliferin-related protein, Mol. Endocrinol., 2, 579-586.

Colosi, P., G. Thordarson, R. Hellmiss, K. Singh, I.A. Forsyth, P. Gluckman and W.I. Wood (1989) Cloning and expression of ovine placental lactogen, Mol. Endocrinol., 3, 1462-1469.

Connor, A.M., P. Waterhouse, R. Khokha and D.T. Denhardt (1989) Characterization of a mouse mitogen-regulated protein/proliferin gene and its promoter: a member of the growth hormone/prolactin gene superfamily, Biochim. Biophys. Acta, 1009, 75-82.

Cooke, N.E., C. Szpirer and G. Levan (1986) The related genes encoding growth hormone and prolactin have been dispersed to chromosomes 10 and 17 in the rat, Endocrinology, 119, 2451-2454.

Cooke, N.E., J. Ray, J.G. Emery and S.A. Liebhaber (1988) Two distinct species of human growth hormone-variant mRNA in the human placenta predict the expression of novel growth hormone proteins, J. Biol. Chem., 263, 9001-9006.

Crister, E.B., J.J. Rutledge and L.R. French (1980) Role of the uterus and the conceptus in regulating luteal lifespan in the mouse, Biosci. Rep., 23, 558-562.

Croze, F., T.G. Kennedy, I.C. Schroedter and H.G. Friesen (1990) Expression of rat prolactin-like protein B in deciduoma of pseudopregnant rat and in decidua during early pregnancy, Endocrinology, 127, 2665-2672.

D'Andrea, A.D., H.F. Lodish and G.G. Wong (1989) Expression cloning of the murine erythropoietin receptor, Cell, 57, 277-285.

Daughaday, W.H., B. Trivedi and B.A. Andrews (1987) The ontogeny of serum GH binding protein in man: a possible indicator of hepatic GH receptor development, J. Clin. Endocrinol. Metab., 65, 1072-1074.

Day, J.R., L.M. Ogren and F. Talamantes (1986) The effect of hypophysectomy on serum placental lactogen and progesterone in the mouse, Endocrinology, 119, 898-903.

Deb, S., T. Youngblood, A.B. Rawitch and M.J. Soares (1989) Placental prolactin-like protein A. Identification and characterization of two major glycoprotein species with antipeptide antibodies, J. Biol. Chem., 264, 14348-14353.

Deb, S., T.N. Faria, K.F. Roby, D. Larsen, S.C. Kwok, F. Talamantes and M.J. Soares (1991a) Identification and characterization of a new member of the prolactin family, placental lactogen-I variant, J. Biol. Chem., 266, 1605-1610.

Deb, S., K.F. Roby, T.N. Faria, D. Larsen and M.J. Soares (1991b) Identification and immunochemical characterization of a major placental secretory protein related to the prolactin-growth hormone family, prolactin-like protein-C, Endocrinology, 128, 3066-3072.

Deb, S., K.F. Roby, T.N. Faria, C. Szpirer, G. Levan, S.C. Kwok and M.J. Soares (1991c)

Molecular cloning and characterization of prolactin-like protein C complementary deoxyribonucleic acid, J. Biol. Chem., 266, 23027-23032.

Deb, S., G.P. Hamlin, K.F. Roby, S.C. Kwok and M.J. Soares (1993) Heterologous expression and characterization of prolactin-like protein-A. Identification of serum binding proteins, J. Biol. Chem., 268, 3298-3305.

Deb, S. and M.J. Soares (1990) Characterization of placental prolactin-like protein-A in intracellular and extracellular compartments, Mol. Cell Endocrinol., 74, 163-172.

Deb, S. and M.L. Soares (1990) Prolactin-like protein-A circulates as biologically latent disulfide-linked high molecular weight complexes, Program of the 72rd Annual Meeting of The Endocrine Society, p114.(Abstract)

Desoye, G., M.O. Schweditsch, K.P. Pfeiffer, R. Zechner and G.M. Kostner (1987) Correlation of hormones with lipid and lipoprotein levels during normal pregnancy and postpartum, J. Clin. Endocrinol. Metab., 64, 704-712.

DiMattia, G.E., B. Gellersen, M.L. Duckworth and H.G. Friesen (1990) Human prolactin gene expression. The use of an alternative noncoding exon in decidua and the IM-9-P3 lymphoblast cell line, J. Biol. Chem., 265, 16412-16421.

Duckworth, M.L., K.L. Kirk and H.G. Friesen (1986a) Isolation and identification of a cDNA clone of rat placental lactogen II, J. Biol. Chem., 261, 10871-10878.

Duckworth, M.L., L.M. Peden and H.G. Friesen (1986b) Isolation of a novel prolactin-like cDNA clone from developing rat placenta, J. Biol. Chem., 261, 10879-10884.

Duckworth, M.L., L.M. Peden and H.G. Friesen (1988) A third prolactin-like protein expressed by the developing rat placenta: complementary deoxyribonucleic acid sequence and partial structure of the gene, Mol. Endocrinol., 2, 912-920.

Duckworth, M.L., I.C. Schroedter and H.G. Friesen (1990) Cellular localization of rat placental lactogen II and rat prolactin-like proteins A and B by in situ hybridization, Placenta., 11, 143-155.

Duello, T.M., J.C. Byatt and R.D. Bremel (1986) Immunohistochemical localization of placental lactogen in binucleate cells of bovine placentomes, Endocrinology, 119, 1351-1355.

Ebbitt, D.M., W.L. Hurley, M.A. Kessler, D.J. McDonald and L.A. Schuler (1989) Characterization of the gene corresponding to bovine placental prolactin-related cDNA I: evolutionary implications, DNA, 8, 161-169.

Edery, M., C. Jolicoeur, C. Levi Meyrueis, I. Dusanter Fourt, B. Petridou, J.M. Boutin, L. Lesueur, P.A. Kelly and J. Djiane (1989) Identification and sequence analysis of a second form of prolactin receptor by molecular cloning of complementary DNA from rabbit mammary gland, Proc. Natl. Acad. Sci. U. S. A., 86, 2112-2116.

English, A., P.J. Fielder, G. Thordarson and F. Talamantes (1991) Synthesis of insulin-like growth factor binding proteins (IGFBPs) by mouse mammary epithelial cells: concomitant hormonal stimulation of IGFBPs and α-lactolbumin, Program of the 73rd Annual Meeting of The Endocrine Society, Washington, DC, 481.(Abstract)

Fahraeus, L., U. Larsson Cohn and L. Wallentin (1985) Plasma lipoproteins including high density lipoprotein subfractions during normal pregnancy, Obstet. Gynecol., 66, 468-472.

Faria, T.N., S. Deb, S.C. Kwok, F. Talamantes and M.J. Soares (1990a) Ontogeny of placental lactogen-I and placental lactogen-II expression in the developing rat placenta, Dev. Biol., 141, 279-291.

Faria, T.N., S. Deb, S.C. Kwok, M. Vandeputte, F. Talamantes and M.J. Soares (1990b) Transplantable rat choriocarcinoma cells express placental lactogen: identification of placental lactogen-I immunoreactive protein and messenger ribonucleic acid, Endocrinology, 127, 3131-3137.

Faria, T.N. and M.J. Soares (1991) Trophoblast cell differentiation: establishment, characterization, and modulation of a rat trophoblast cell line expressing members of the placental prolactin family, Endocrinology, 129, 2895-2906.

Fielder, P.J. and F. Talamantes (1987) The lipolytic effects of mouse placental lactogen II, mouse prolactin, and mouse growth hormone on adipose tissue from virgin and pregnant mice, Endocrinology, 121, 493-497.

Fitzpatrick, S.L., W.H. Walker and G.F. Saunders (1990) DNA sequences involved in the transcriptional activation of a human placental lactogen gene, Mol. Endocrinol., 4, 1815-1826.

Frankenne, F., F. Rentier Delrue, M.L. Scippo, J. Martial and G. Hennen (1987) Expression of the growth hormone variant gene in human placenta, J. Clin. Endocrinol. Metab., 64, 635-637.

Frankenne, F., J. Closset, F. Gomez, M.L. Scippo, J. Smal and G. Hennen (1988) The physiology of growth hormones (GHs) in pregnant women and partial characterization of the placental GH variant, J. Clin. Endocrinol. Metab., 66, 1171-1180.

Freemark, M., M. Comer and S. Handwerger (1986) Placental lactogen and GH receptors in

sheep liver: striking differences in ontogeny and function, Am. J. Physiol, 251, E328-E333.

Freemark, M., M. Comer, G. Korner and S. Handwerger (1987) A unique placental lactogen receptor: implications for fetal growth, Endocrinology, 120, 1865-1872.

Freemark, M., M. Comer and G. Korner (1988) Differential solubilization of placental lactogen (PL)- and growth hormone-binding sites: further evidence for a unique PL receptor in fetal and maternal liver, Endocrinology, 122, 2771-2779.

Freemark, M., M. Comer, T. Mularoni, A.J. D'Ercole, A. Grandis and L. Kodack (1989) Nutritional regulation of the placental lactogen receptor in fetal liver: implications for fetal metabolism and growth, Endocrinology, 125, 1504-1512.

Freemark, M., M. Comer, T. Mularoni, A.J. D'Ercole, A. Grandis and L. Kodack (1990) Placental lactogen receptors in maternal sheep liver: effects of fasting and refeeding, Am. J. Physiol, 258, E338-E346.

Freemark, M., A. Keen, J. Fowlkes, T. Mularoni, M. Comer, A. Grandis and L. Kodack (1992) The placental lactogen receptor in maternal and fetal sheep liver: regulation by glucose and role in the pathogenesis of fasting during pregnancy, Endocrinology, 130, 1063-1070.

Freemark, M. and M. Comer (1989) Purification of a distinct placental lactogen receptor, a new member of the growth hormone/prolactin receptor family, J. Clin. Invest, 83, 883-889.

Freemark, M. and S. Handwerger (1984) Ovine placental lactogen stimulates glycogen synthesis in fetal rat hepatocytes, Am. J. Physiol, 246, E21-E24.

Freemark, M. and S. Handwerger (1986) The glycogenic effects of placental lactogen and growth hormone in ovine fetal liver are mediated through binding to specific fetal ovine placental lactogen receptors, Endocrinology, 118, 613-618.

Freemark, M. and S. Handwerger (1989) The role of placental lactogen in the regulation of fetal metabolism and growth, J. Pediatr. Gastroenterol. Nutr., 8, 281-283.

Freinkel, N., B.E. Metzger, R.L. Phelps, S.L. Dooley, E.S. Ogata, R.M. Radvany and A. Belton (1985) Gestational diabetes mellitus. Heterogeneity of maternal age, weight, insulin secretion, HLA antigens, and islet cell antibodies and the impact of maternal metabolism on pancreatic B-cell and somatic development in the offspring, Diabetes, 34 Suppl 2, 1-7.

Friesen, H.G., M.C. Robertson, M.L. Duckworth, I. Schroedter, N. Quan and J-C. Vuille (1993) Placental lactogen/growth hormone gene family, in: P.C.K. Leung, A.J.W. Hsueh and H.G. Friesen (Eds.), , Springer-Verlag New York Inc. , New York, pp. 171-180.

Fukunaga, R., E. Ishizaka Ikeda, Y. Seto and S. Nagata (1990) Expression cloning of a receptor for murine granulocyte colony-stimulating factor, Cell, 61, 341-350.

Galosy, S.S., A. Gertler, G. Elberg and D.M. Laird (1991) Distinct placental lactogen and prolactin (lactogen) receptors in bovine endometrium, Mol. Cell Endocrinol., 78, 229-236.

Gearing, D.P., J.A. King, N.M. Gough and N.A. Nicola (1989) Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor, EMBO J., 8, 3667-3676.

Glaser, L.A., P.A. Kelly and G. Gibori (1984) Differential action and secretion of rat placental lactogens, Endocrinology, 115, 969-976.

Golander, A., T. Hurly, J. Barrett, A. Hize and S. Handwerger (1978) Prolactin synthesis by human chorion-decidual tissue: a possible source of amniotic fluid prolactin, Science, 202, 311-313.

Golander, A., T. Hurly, J. Barrett and S. Handwerger (1979) Synthesis of prolactin by human decidua in vitro, J. Endocrinol., 82, 263-267.

Golander, A., R. Richards, K. Thrailkill, D. Capel, D. Rogers and S. Handwerger (1988) Decidual prolactin (PRL)-releasing factor stimulates the synthesis of PRL from human decidual cells, Endocrinology, 123, 335-339.

Goodwin, R.G., D. Friend, S.F. Ziegler, R. Jerzy, B.A. Falk, S. Gimpel, D. Cosman, S.K. Dower, C.J. March, A.E. Namen and et al (1990) Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily, Cell, 60, 941-951.

Grandis, A., V. Jorgensen, L. Kodack, S. Quarfordt and S. Handwerger (1989) High-density lipoproteins (HDL) stimulate placental lactogen secretion in pregnant ewes: further evidence for a role of HDL in placental lactogen secretion during pregnancy, J. Endocrinol., 120, 423-427.

Grattan, D.R. and R.L. Averill (1990) Effect of ovarian steroids on a nocturnal surge of prolactin secretion that precedes parturition in the rat, Endocrinology, 126, 1199-1205.

Grumbach, M.M., S.L. Kaplan, J.J. Sciarra and I.M Burr (1968) Chorionic growth hormone-prolactin (CGP): secretion, disposition, biologic activity in man, and postulated function as the "growth hormone" of the second half of pregnancy, Ann. N. Y. Acad. Sci., 148, 501-508.

Grumbach, M.M. and S.L. Kaplan (1964) On the placental origin and purification of chorionic "growth hormone-prolacin" and its immunoassay in pregnancy, Trens. N. Y. Acad. Sci., 27, 167-172.

Guo, X.J., A.F. Chambers, C.L. Parfett, P. Waterhouse, L.C. Murphy, R.E. Reid, A.M. Craig, D.R. Edwards and D.T. Denhardt (1990) Identification of a serum-inducible messenger RNA (5B10) as the mouse homologue of calcyclin: tissue distribution and expression in metastatic, ras-transformed NIH 3T3 cells, Cell Growth Differ., 1, 333-338.

Hall, K., G. Enberg, E. Hellem, G. Lundin, A. Ottosson Seeberger, V. Sara, O. Trygstad and U. Ofverholm (1984) Somatomedin levels in pregnancy: longitudinal study in healthy subjects and patients with growth hormone deficiency, J. Clin. Endocrinol. Metab., 59, 587-594.

Handwerger, S., J. Barrett, L. Tyrey and D. Schomberg (1973) Differential effect of cyclic adenosine monophosphate on the secretion of human placental lactogen and human chorionic gonadotropin, J. Clin. Endocrinol. Metab., 36, 1268-1279.

Handwerger, S., D. Capel, G. Korner and R. Richards (1987a) Purification of decidual prolactin-releasing factor, a placental protein that stimulates prolactin release from human decidual tissue, Biochem. Biophys. Res. Commun., 147, 452-459.

Handwerger, S., S. Quarfordt, J. Barrett and I. Harman (1987b) Apolipoproteins AI, AII, and CI stimulate placental lactogen release from human placental tissue. A novel action of high density lipoprotein apolipoproteins, J. Clin. Invest, 79, 625-628.

Handwerger, S., E. Markoff and R. Richards (1991a) Regulation of the synthesis and release of decidual prolactin by placental and autocrine/paracrine factors, Placenta., 12, 121-130.

Handwerger, S., R. Richards and E. Markoff (1991b) Autocrine/paracrine regulation of prolactin release from human decidual cells, Ann. N. Y. Acad. Sci., 622, 111-119.

Harigaya, T., W.C. Smith and F. Talamantes (1988) Hepatic placental lactogen receptors during pregnancy in the mouse, Endocrinology, 122, 1366-1372.

Hartmann, D.P., J.W. Holaday and E.W. Bernton (1989) Inhibition of lymphocyte proliferation by antibodies to prolactin, FASEB J., 3, 2194-2202.

Harvey, C., S.M. Jackson, S.K. Siddiqui and A. Gutierrez Hartmann (1991) Structure-function analysis of the rat prolactin promoter: phasing requirements of proximal cell-specific elements, Mol. Endocrinol., 5, 836-843.

Hatakeyama, M., M. Tsudo, S. Minamoto, T. Kono, T. Doi, T. Miyata, M. Miyasaka and T. Taniguchi (1989) Interleukin-2 receptor beta chain gene: generation of three receptor forms

by cloned human alpha and beta chain cDNA's, Science, 244, 551-556.

Healy, D.L. and G.D. Hodgen (1983) The endocrinology of human endometrium, Obstet. Gynecol. Surv., 38, 509-530.

Herrington, A.C., J. Graham and D.L. Healy (1980) The presence of lactogen receptors in human chorion laeve, J. Clin. Endocrinol. Metab., 51, 1466-1475.

Hershman, J.M., A. Kojima and H.G. Friesen (1973) Effect of thyrotropin-releasing hormone on human pituitary thyrotropin, prolactin, placental lactogen and chorionic thyrotropin, J. Clin. Endocrinol. Metab., 36, 497-503.

Herz, Z., I. Khan, P.G. Jayatilak and G. Gibori (1986) Evidence for the secretion of decidual luteotropin: a prolactin-like hormone produced by rat decidual cells, Endocrinology, 118, 2203-2209.

Hill, D.J., C.J. Crace and R.D. Milner (1985) Incorporation of [3H]thymidine by isolated fetal myoblasts and fibroblasts in response to human placental lactogen (HPL): possible mediation of HPL action by release of immunoreactive SM-C, J. Cell Physiol, 125, 337-344.

Hill, D.J., C.J. Crace, A.J. Strain and R.D. Milner (1986) Regulation of amino acid uptake and deoxyribonucleic acid synthesis in isolated human fetal fibroblasts and myoblasts: effect of human placental lactogen, somatomedin-C, multiplication-stimulating activity, and insulin, J. Clin. Endocrinol. Metab., 62, 753-760.

Hill, D.J., M. Freemark, A.J. Strain, S. Handwerger and R.D. Milner (1988) Placental lactogen and growth hormone receptors in human fetal tissues: relationship to fetal plasma human placental lactogen concentrations and fetal growth, J. Clin. Endocrinol. Metab., 66, 1283-1290.

Hocquette, J.F., M.C. Postel Vinay, J. Djiane, A. Tar and P.A. Kelly (1990) Human liver growth hormone receptor and plasma binding protein: characterization and partial purification, Endocrinology, 127, 1665-1672.

Hughes, J.P. and H.G. Friesen (1985) The nature and regulation of the receptors for pituitary growth hormone, Annu. Rev. Physiol, 47, 469-482.

Hurley, T.W., A.J. D'Ercole, S. Handwerger, L.E. Underwood, R.W. Fulanetto and R.E. Fellows (1977) Ovine placnetal lactogen induces somatomedin: a possible role in fetal growth, Endocrinology, 101, 1635-1642.

Hwang, P., J.B. Murray, J.W. Jacobs, H.D. Niall and H.G. Friesen (1974) Human amniotic fluid prolactin: purification by affinity chromotography and amino-terminal sequence,

Biochemistry, 13, 2354-2361.

Ingraham, H.A., S.E. Flynn, J.W. Voss, V.R. Albert, M.S. Kapiloff, L. Wilson and M.G. Rosenfeld (1990) The POU-specific domain of Pit-1 is essential for sequence-specific, high affinity DNA binding and DNA-dependent Pit-1-Pit-1 interactions, Cell, 61, 1021-1033.

Itoh, N., S. Yonehara, J. Schreurs, D.M. Gorman, K. Maruyama, A. Ishii, I. Yahara, K. Arai and A. Miyajima (1990) Cloning of an interleukin-3 receptor gene: a member of a distinct receptor gene family, Science, 247, 324-327.

Jackson, L.L., P. Colosi, F. Talamantes and D.I. Linzer (1986) Molecular cloning of mouse placental lactogen cDNA, Proc. Natl. Acad. Sci. U. S. A., 83, 8496-8500.

Jasimovich, J.B., K. Merisko and L. Boccella (1977) Amniotic prolactin control over amniotic and fetal extracellular fluid water and electrolytes in the rhesus monkey, Endocrinology, 100, 564-570.

Jayatilak, P.G., L.A. Glaser, R. Basuray, P.A. Kelly and G. Gibori (1985) Identification and partial characterization of a prolactin-like hormone produced by rat decidual tissue, Proc. Natl. Acad. Sci. U. S. A., 82, 217-221.

Jayatilak, P.G., T.K. Puryear, Z. Herz, A. Fazleabas and G. Gibori (1989) Protein secretion by mesometrial and antimesometrial rat decidual tissue: evidence for differential gene expression, Endocrinology, 125, 659-666.

Kaplan, S.L. and M.M. Grumbach (1981) Chorionic somatomammotropin in primates: secretion and physiology, in: J. Resko and M. Novy (Eds.), Fetal Endocrinology, Academic Press, New York, pp. 127-135.

Karin, M., J.L. Castrillo and L.E. Theill (1990) Growth hormone gene regulation: a paradigm for cell-type-specific gene activation, Trends. Genet., 6, 92-96.

Kauma, S. and S.S. Shapiro (1986) Immunoperoxidase localization of prolactin in endometrium during normal menstrual, luteal phase defect, and corrected luteal phase defect cycles, Fertil. Steril., 46, 37-41.

Keech, C.A. and A. Gutierrez Hartmann (1989) Analysis of rat prolactin promoter sequences that mediate pituitary-specific and 3',5'-cyclic adenosine monophosphate-regulated gene expression in vivo, Mol. Endocrinol., 3, 832-839.

Kelly, P.A., T. Tsushima, P.R.C. Shiu and H.G. Friesen (1976) Lactogenic and growth hormone-like activities in pregnancy determined by radioreceptor assay, Endocrinology, 99, 765-771.

Kelly, P.A., J. Djiane, M.C. Postel Vinay and M. Edery (1991) The prolactin/growth hormone receptor family, Endocr. Rev., 12, 235-251.

Kessler, M.A., M. Milosavljevic, C.G. Zieler and L.A. Schuler (1989) A subfamily of bovine prolactin-related transcripts distinct from placental lactogen in the fetal placenta, Biochemistry, 28, 5154-5161.

Kessler, M.A. and L.A. Schuler (1991) Structure of the bovine placental lactogen gene and alternative splicing of transcripts, DNA Cell Biol., 10, 93-104.

Kishi, K., L. Ogren, J.N. Southard and F. Talamantes (1988) Pituitary factors regulating mouse placental lactogen-II secretion during the last half of pregnancy in mice, Endocrinology, 122, 2309-2317.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T-lambda, Nature, 227, 680-687.

Lee, S.J., F. Talamantes, E. Wilder, D.I. Linzer and D. Nathans (1988) Trophoblastic giant cells of the mouse placenta as the site of proliferin synthesis, Endocrinology, 122, 1761-1768.

Lee, S.J. and D. Nathans (1987) Secretion of proliferin, Endocrinology, 120, 208-213.

Lee, S.J. and D. Nathans (1988) Proliferin secreted by cultured cells binds to mannose 6-phosphate receptors, J. Biol. Chem., 263, 3521-3527.

Leidgi, F., Shepard, A.R., Zhang, W., Stelter, A., Cattini, P.A., Baxter, J.D., Eberhardt, N.L. (1992) Thyroid hormone responsiveness in human growth hormone-related genes, J. Biol. Chem., 267, 913-921.

Lemaigre, F.P., B. Peers, D.A. Lafontaine, M. Mathy Hartert, G.G. Rousseau, A. Belayew and J.A. Martial (1989) Pituitary-specific factor binding to the human prolactin, growth hormone, and placental lactogen genes, DNA, 8, 149-159.

Leung, D.W., S.A. Spencer, G. Cachianes, R.G. Hammonds, C. Collins, W.J. Henzel, R. Barnard, M.J. Waters and W.I. Wood (1987) Growth hormone receptor and serum binding protein: purification, cloning and expression, Nature, 330, 537-543.

Levan, G., J. Szpirer, C. Szpirer, K. Klinga, C. Hanson and M.Q. Islam (1991) The gene map of the Norway rat (Rattus norvegicus) and comparative mapping with mouse and man, Genomics, 10, 699-718.

Linkie, D.M. and G.D. Niswender (1973) Characterization of rat placental luteotropin: physiological and physiochemical preperties, Biosci. Rep., 8, 48-54.

Linzer, D.I., S.J. Lee, L. Ogren, F. Talamantes and D. Nathans (1985) Identification of proliferin mRNA and protein in mouse placenta, Proc. Natl. Acad. Sci. U. S. A., 82, 4356-4359.

Linzer, D.I. and D. Nathans (1984) Nucleotide sequence of a growth-related mRNA encoding a member of the prolactin-growth hormone family, Proc. Natl. Acad. Sci. U. S. A., 81, 4255-4259.

Linzer, D.I. and D. Nathans (1985) A new member of the prolactin-growth hormone gene family expressed in mouse placenta, EMBO J., 4, 1419-1423.

Lytras, A., N. Quan, M.E. Vrontakis, J.E. Shaw, P.A. Cattini and H.G. Friesen (1993) Growth hormone expression in human Burkitt lymphoma serum-free Ramos cell line, Endocrinology, 132, 620-628.

MacLeod, J.N., Lee, A.K., Liebhaber, S.A., Cooke, N.E. (1992) Developmental control and alternative splicing of the placnetally expressed transcripts from the human growth hormone gene cluster, J. Biol. Chem., 267, 14219-14226.

Maurer, R.A., K.E. Kim, R.N. Day and A.C. Notides (1990) Regulation of prolactin gene expression by estradiol, Prog. Clin. Biol. Res., 322, 159-169.

Maurer, R.A. and A.C. Notides (1987) Identification of an estrogen-responsive element from the 5'-flanking region of the rat prolactin gene, Mol. Cell Biol., 7, 4247-4254.

McCoshen, J.A. and J. Barc (1985) Prolactin bioactivity following decidual synthesis and transport by amniochorion, Am. J. Obstet. Gynecol., 153, 217-223.

Miller, W.L. and N.L. Eberhardt (1983) Structure and evolution of the growth hormone gene family, Endocr. Rev., 4, 97-130.

Mordacq, J.C. and D.I. Linzer (1989) Co-localization of elements required for phorbol ester stimulation and glucocorticoid repression of proliferin gene expression, Genes Dev., 3, 760-769.

Morishige, M.K. and I. Rothchild (1974) Temporal aspects of the regulation of corpus luteum function by luteinizing hormone prolactin and placental luteotropin during the first half of pregnancy in the rat, Endocrinology, 95, 260-265.

Mosley, B., M.P. Beckmann, C.J. March, R.L. Idzerda, S.D. Gimpel, T. VandenBos, D.

Friend, A. Alpert, D. Anderson, J. Jackson and et al (1989) The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms, Cell, 59, 335-348.

Mukherjee, P., A.M. Mastro and W.C. Hymer (1990) Prolactin induction of interleukin-2 receptors on rat splenic lymphocytes, Endocrinology, 126, 88-94.

Mulder, G.H. (1989) Effects of decidual prolactin on amino-chorion water permeability, in: O. Genbacev, A. Klopper and R. Beaconsfield (Eds.), Placenta as a model and a source, Plenum Press, New York, pp. 103-112.

Nagy, E., I. Berczi, G.E. Wren, S.L. Asa and K. Kovacs (1983) Immunomodulation by bromocriptine, Immunopharmacology, 6, 231-243.

Newton, W.H. and N. Beck (1939) Placental activity in the mouse in the absence of the pituitary gland, J. Endocrinol., 1, 65-71.

Niall, H.D., M.L. Hodan, R. Sauer, I.Y. Rosenblum and F.C. Greenwood (1971) Sequences of pituitary and placnetal lactogenic and growth hormones: evolution from a primordial peptide by gene duplication, Proc. Natl. Acad. Sci. U. S. A., 68, 866-871.

Nickle, B.E. and Cattini, P.A. (1991) Tissue-specific expression and thyroid hormone regulation of the endogenous placental growth hormone variant and chorionic somatomammotropin genes in a human choriocarcinoma cell line, Endocrinology, 128, 2353-2359.

Nickle, B.E., Bock, M.E., Nachtigal, M.W. and Cattni, P.A. (1993) Differential expression of human placnetal growth hormone variant aand chorionic somatomammotropin genes in choriocarcinoma cell treated with methotrexate, Mol. Cell. End. 91, 159-166.

Nicoll, C.S., G.L. Mayer and S.M. Russell (1986) Structural features of prolactins and growth hormones that can be related to their biological properties [published erratum appears in Endocr Rev 1987 Feb;8(1):43], Endocr. Rev., 7, 169-203.

Nielsen, P.V., H. Pederson and E.M. Kampmann (1979) Absence of human placental lactogen in an otherwise normal pregnancy, Am. J. Obstet. Gynecol., 135, 322-331.

Niven, P.A.R., W.C. Buli and W.N. Spellacy (1974) The effect of intravenous estrogen injection on plasma human placental lactogen levels, J. Obstet. Gonecol. Br. Comm., 81, 466-472.

Ogilvie, S., W.C. Buhi, J.A. Olson and K.T. Shiverick (1990a) Identification of a novel family of growth hormone-related proteins secreted by rat placenta, Endocrinology, 126,

3271-3273.

Ogilvie, S., M.L. Duckworth, L.H. Larkin, W.C. Buhi and K.T. Shiverick (1990b) De novo synthesis and secretion of prolactin-like protein-B by rat placental explants, Endocrinology, 126, 2561-2566.

Ogren, L., J.N. Southard, P. Colosi, D.I. Linzer and F. Talamantes (1989) Mouse placental lactogen-I: RIA and gestational profile in maternal serum, Endocrinology, 125, 2253-2257.

Ogren, L. and F. Talamantes (1988) Prolactins of pregnancy and their cellular source, Int. Rev. Cytol., 112, 1-65.

Ormandy, C.J., C.L. Clarke and R.L. Sutherland (1990) Solubilization and characterization of a lactogenic receptor from human placental chorion membranes, J. Cell Biochem., 43, 1-15.

Owerbach, D., W.J. Rutter and J.A. Martial (1980) Genes for growth hormone, chorionic somatomammotropin and growth hormone-like gene on chromosone 17 in humans, Science, 209, 289-292.

Peeters, S. and H.G. Friesen (1977) A growth hormone binding factor in the serum of pregnant mice, Endocrinology, 101, 1164-1168.

Pellegrini, I., J.J. Lebrun, S. Ali and P.A. Kelly (1992) Expression of prolactin and its receptor in human lymphoid cells, Mol. Endocrinol., 6, 1023-1031.

Pencharz, R.I. and J.A. Long (1931) The effect of hypophysectomy on gestation in the rat, Science, 74, 206-209.

Petit, A., G. Guillon, M. Tence, S. Jard, N. Gallo Payet, D. Bellabarba, J.G. Lehoux and S. Belisle (1989) Angiotensin II stimulates both inositol phosphate production and human placental lactogen release from human trophoblastic cells, J. Clin. Endocrinol. Metab., 69, 280-286.

Pinon, I., K. Kishi and F. Talamantes (1988) The kinetics of disappearance of endogenous mouse placental lactogen-II in intact and hypophysectomized pregnant mice, Mol. Cell Endocrinol., 55, 45-51.

Richards, R.G., S. Basavappa and D. Rogers (1990) Long term effects of apolipoprotein AI on the synthesis and release of placental lactogen from human trophoblast cells, 72nd Annual Meeting of The Endocrine Society, 115.(Abstract)

Richards, S.R., M.H. Kim and W.B. Malarkey (1982) Evidence that chorion-decidual

prolactin release is calcium depedent, J. Clin. Endocrinol. Metab., 54, 820-823.

Robertson, M.C., B. Gillespie and H.G. Friesen (1982) Characterization of the two forms of the rat placental lactogen (rPL): rPL-I and rPL-II, Endocrinology, 1862, 1869.

Robertson, M.C., R.E. Owens, J. Klindt and H.G. Friesen (1984a) Ovariectomy leads to a rapid increase in rat placental lactogen secretion, Endocrinology, 114, 1805-1811.

Robertson, M.C., R.E. Owens, J.A. McCoshen and H.G. Friesen (1984b) Ovarian factors inhibit and fetal factors stimulate the secretion of rat placental lactogen, Endocrinology, 114, 22-30.

Robertson, M.C., F. Croze, I.C. Schroedter and H.G. Friesen (1990) Molecular cloning and expression of rat placental lactogen-I complementary deoxyribonucleic acid, Endocrinology, 127, 702-710.

Robertson, M.C., I.C. Schroedter and H.G. Friesen (1991) Molecular cloning and expression of rat placental lactogen-Iv, a variant of rPL-I present in late pregnant rat placenta, Endocrinology, 129, 2746-2756.

Robertson, M.C. and H.G. Friesen (1981) Two forms of rat placental lactogen revealed by radioimmunoassay, Endocrinology, 108, 2388-2393.

Rogers, B.L., M.G. Sobnosky and G.F. Saunders (1986) Transcriptional enhancer within the human placental lactogen and growth hormone multigene cluster, Nucleic Acids Res., 14, 7647-7659.

Rohringer, R. and D.W. Holden (1985) Protein blotting: detection of proteins with colloidal gold, and of glycoproteins and lectins with biotin-conjugated and enzyme probes, Anal. Biochem., 144, 118-127.

Rosenberg, S.M. and A.S. Bhatnagar (1984) Sex steroid and human chorionic gonadotropin modulation of in vitro prolactin production by human term decidua, Am. J. Obstet. Gynecol., 148, 461-465.

Sadeghi, H., B.S. Wang, A.L. Lumanglas, J.S. Logan and W.R. Baumbach (1990) Identification of the origin of the growth hormone-binding protein in rat serum, Mol. Endocrinol., 4, 1799-1805.

Salacinski, P.R.P., C. McLean, J.E.C. Sykes and V.V. Clement-Jones (1981) Iodination of proteins, glycoproteins and peptide by a solid-phase oxidazing agent 1,3,4,6,-tetracholo-3α glycoluri (Iodogen), Anal. Biochem., 117, 136-142.

Schuler, L.A., K. Shimomura, M.A. Kessler, C.G. Zieler and R.D. Bremel (1988) Bovine placental lactogen: molecular cloning and protein structure, Biochemistry, 27, 8443-8448.

Schuler, L.A. and W.L. Hurley (1987) Molecular cloning of a prolactin-related mRNA expressed in bovine placenta, Proc. Natl. Acad. Sci. U. S. A., 84, 5650-5654.

Shida, M.M., L.L. Jackson Grusby, S.R. Ross and D.I. Linzer (1992) Placental-specific expression from the mouse placental lactogen II gene promoter, Proc. Natl. Acad. Sci. U. S. A., 89, 3864-3868.

Shine, J., P.H. Seeburg and J.A. Martial (1977) Construction and analysis of recombinant DNA for huamn chionic somatommamatropin, Nature, 270, 494-499.

Shirota, M., D. Banville, S. Ali, C. Jolicoeur, J.M. Boutin, M. Edery, J. Djiane and P.A. Kelly (1990) Expression of two forms of prolactin receptor in rat ovary and liver, Mol. Endocrinol., 4, 1136-1143.

Shiu, P.R.C., P.A. Kelly and H.G. Friesen (1973) Radioreceptor assay for prolactin and other lactogenic hormones, Science, 180, 968-969.

Sideri, M., G. De Virgiliis, F. Guidobono, N. Borgese, L.P. Sereni, U. Nicolini and G. Remotti (1983) Immunologically undetectable human placental lactogen in a normal pregnancy. Case report, Br. J. Obstet. Gynaecol., 90, 771-773.

Smith, D.B. and K.S. Johnson (1988) Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase, Gene, 67, 31-40.

Smith, W.C. and F. Talamantes (1987) Identification and characterization of a heterogeneous population of growth hormone receptors in mouse hepatic membranes, J. Biol. Chem., 262, 2213-2219.

Soares, M.J., T.N. Faria, K.F. Roby and S. Deb (1991) Pregnancy and the prolactin family of hormones: coordination of anterior pituitary, uterine, and placental expression, Endocr. Rev., 12, 402-423.

Soares, M.J. and F. Talamantes (1985) Placental lactogen secretion in the mouse: in vitro responses and ovarian and hormonal influences, J. Exp. Zool., 234, 97-104.

Southard, J.N., G.T. Campbell and F. Talamantes (1987) Hamster placental lactogens: gestational profiles and high molecular weight forms, Endocrinology, 121, 900-906.

Southard, J.N., L. Do, W.C. Smith and F. Talamantes (1989) Hamster placental lactogen-II contains a structural feature unique among the growth hormone-prolactin-placental lactogen

family, Mol. Endocrinol., 3, 1710-1713.

Southard, J.N. and F. Talamantes (1989) High molecular weight forms of placental lactogen: evidence for lactogen-macroglobulin complexes in rodents and humans, Endocrinology, 125, 791-800.

Strain, A.J., D.J. Hill, I. Swenne and R.D. Milner (1987) Regulation of DNA synthesis in human fetal hepatocytes by placental lactogen, growth hormone, and insulin-like growth factor I/somatomedin-C, J. Cell Physiol, 132, 33-40.

Swenne, I., D.J. Hill, A.J. Strain and R.D. Milner (1987) Effects of human placental lactogen and growth hormone on the production of insulin and somatomedin C/insulin-like growth factor I by human fetal pancreas in tissue culture, J. Endocrinol., 113, 297-303.

Takaki, S., A. Tominaga, Y. Hitoshi, S. Mita, E. Sonoda, N. Yamaguchi and K. Takatsu (1990) Molecular cloning and expression of the murine interleukin-5 receptor, EMBO J., 9, 4367-4374.

Tanaka, T., P.R.C. Shiu, P.W. Gout, C.T. Beer, R.L. Nobel and H.G. Friesen (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.

Thordarson, G., R. Villalobos, P. Colosi, J. Southard, L. Ogren and F. Talamantes (1986) Lactogenic response of cultured mouse mammary epithelial cells to mouse placental lactogen, J. Endocrinol., 109, 263-274.

Thordarson, G., G.H. McDowell, S.V. Smith, S. Iley and I.A. Forsyth (1987) Effects of continuous intravenous infusion of an ovine placental extract enriched in placental lactogen on plasma hormones, metabolites and metabolite biokinetics in non-pregnant sheep, J. Endocrinol., 113, 277-283.

Thordarson, G., J.N. Southard and F. Talamantes (1991) Purification and characterization of mouse decidual calcyclin: a novel stimulator of mouse placental lactogen-II secretion, Endocrinology, 129, 1257-1265.

Thordarson, G. and F. Talamantes (1987) Role of the placenta in mammary gland development and function, in: M.C. Neville and C. Daniel (Eds.), The Mammary Gland: Development, Regulation, and Function, Plenum Press, New York, pp. 459-471.

Thoreau, E., B. Petridou, P.A. Kelly, J. Djiane and J.P. Mornon (1991) Structural symmetry of the extracellular domain of the cytokine/growth hormone/prolactin receptor family and interferon receptors revealed by hydrophobic cluster analysis, FEBS Lett., 282, 26-31.

Thrailkill, K.M., A. Golander, L.E. Underwood and S. Handwerger (1988) Insulin-like growth factor I stimulates the synthesis and release of prolactin from human decidual cells, Endocrinology, 123, 2930-2934.

Tomogane, H., A.M. Mistry and J.L. Voogt (1992) Late pregnancy and rat choriocarcinoma cells inhibit nocturnal prolactin surges and serotonin-induced prolactin release, Endocrinology, 130, 23-28.

Tong, Y., J. Simard, C. Labrie, H.F. Zhao, F. Labrie and G. Pelletier (1989) Inhibitory effect of androgen on estrogen-induced prolactin messenger ribonucleic acid accumulation in the male rat anterior pituitary gland, Endocrinology, 125, 1821-1828.

Tong, Y.A., H.F. Zhao, F. Labrie and G. Pelletier (1989) Ontogeny of prolactin mRNA in the rat pituitary gland as evaluated by in situ hybridization, Mol. Cell Endocrinol., 67, 11-16.

Tonkowicz, P.A. and J.L. Voogt (1983a) Examination of rat placental lactogen and prolactin at 6-hr intervals during midpregnancy, Proc. Soc. Exp. Biol. Med., 173, 583-587.

Tonkowicz, P.A. and J.L. Voogt (1983b) Termination of prolactin surges with development of placental lactogen secretion in the pregnant rat, Endocrinology, 113, 1314-1318.

Towbin, H., T. Staehelin and J. Gordon (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A., 76, 4350-4354.

Tsushima, T. and H.G. Friesen (1973) Radioreceptor assay for growth hormone, J. Clin. Endocrinol. Metab., 37, 334-342.

Urbanek, M., J.N. MacLeod, N.E. Cooke and S.A. Liebhaber (1992) Expression of a human growth hormone (hGH) receptor isoform is predicted by tissue-specific alternative splicing of exon 3 of the hGH receptor gene transcript, Mol. Endocrinol., 6, 279-287.

Voogt, J. and W.J. de Greef (1989) Inhibition of nocturnal prolactin surges in the pregnant rat by incubation medium containing placental lactogen, Proc. Soc. Exp. Biol. Med., 191, 403-407.

Voogt, J.L., P.L. Pakrasi, D.C. Johnson and S.K. Dey (1985) Evidence for pituitary regulation of placental lactogen in the rat, J. Endocrinol., 107, 121-126.

Vuille, J-C., Cattini, P.A., Bock, M.E., Verstuyf, A., Schroedter, I.C., Duckworth, M.L. and Friesen, H.G. (1993) Rat prolactin-like protein A partial gene and promoter structure: promoter activity in placental and pituitary cells, Mol. Cell. End. 96, 91-98

Walker, W.H., S.L. Fitzpatrick and G.F. Saunders (1990) Human placental lactogen transcriptional enhancer. Tissue specificity and binding with specific proteins, J. Biol. Chem., 265, 12940-12948.

Welsh, C.W. and M.J. McManus (1977) Stimulation of DNA synthesis by human placental lactogen or insulin in organ culture of benign human breast tumors, Cancer Res., 37, 2257-2262.

Wilder, E.L. and D.I. Linzer (1989) Participation of multiple factors, including proliferin, in the inhibition of myogenic differentiation, Mol. Cell Biol., 9, 430-441.

Wu, Y.Q., E.V. Jorgensen and S. Handwerger (1988) High density lipoproteins stimulate placental lactogen release and adenosine 3',5'-monophosphate (cAMP) production in human trophoblast cells: evidence for cAMP as a second messenger in human placental lactogen release, Endocrinology, 123, 1879-1884.

Yamakawa, M., M. Tanaka, M. Koyama, Y. Kagesato, M. Watahiki, M. Yamamoto and K. Nakashima (1990) Expression of new members of the prolactin growth hormone gene family in bovine placenta. Isolation and characterization of two prolactin-like cDNA clones, J. Biol. Chem., 265, 8915-8920.

Yamasaki, K., T. Taga, Y. Hirata, H. Yawata, Y. Kawanishi, B. Seed, T. Taniguchi, T. Hirano and T. Kishimoto (1988) Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor, Science, 241, 825-828.

Ying, Y.K., D.B. Maier, J.F. Randolph, S. Kuslis and D.H. Riddick (1988) Stimulation of uterine prolactin secretion by human chorionic gonadotropin and progesterone in the cynomolgus monkey, Fertil. Steril., 50, 976-979.

Ymer, S.I. and A.C. Herington (1985) Evidence for the specific binding of growth hormone to a receptor-like protein in rabbit serum, Mol. Cell Endocrinol., 41, 153-161.

Ymer, S.I. and A.C. Herington (1987) Structural studies on membrane-bound and soluble growth-hormone-binding proteins of rabbit liver, Biochem. J., 242, 713-720.

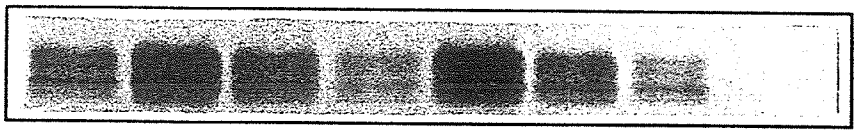
Yu Lee, L.Y., A.M. Stevens, J.A. Hrachovy and L.A. Schwarz (1990) Prolactin-mediated regulation of gene transcription in lymphocytes, Ann. N. Y. Acad. Sci., 594, 146-155.

Zeitler, P., E. Murphy and S. Handwerger (1986) Arachidonic acid stimulates 45calcium efflux and hPL release in isolated trophoblast cells, Life Sci., 38, 99-107.

Zhang, R., E. Buczko, C.H. Tsai Morris, Z.Z. Hu and M.L. Dufau (1990) Isolation and characterization of two novel rat ovarian lactogen receptor cDNA species, Biochem. Biophys.

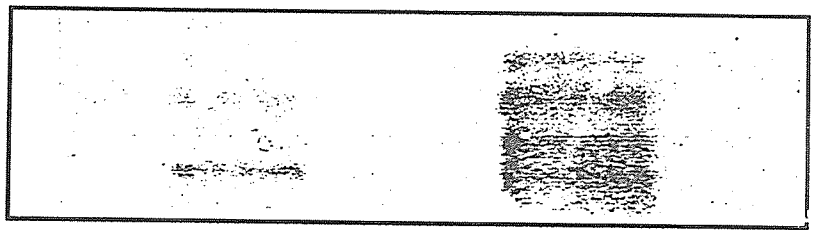
Res. Commun., 168, 415-422.

Figure 3. Immunoprecipitation of [^{35}S] methionine-labelled proteins synthesized in Chinese Hamster Ovary (CHO) cells transfected with an rPLP-A expression clone. The individual clones of rPLP-A transfected CHO cells from 100 μM, 200 μM methotrexate-resistant medium along with untransfected CHO cells were labelled with 100 μCi/ml [^{35}S]-methionine for 8 hr. Equal amounts of [^{35}S] methionine-labelled protein (100,000 cpm of TCA precipitated [^{35}S]methionine-labelled proteins) were immunoprecipitated with rPLP-A antipeptide antiserum, and then electrophoretically separated by 12.5% SDS-PAGE. Lanes 1 to 6 are clones selected on 200 μM MTX, lane 7 is selected on 100 μM, lane 8 is medium from parental DG44 cells. The gel was dried and then exposed to Kodak X-Omat AR X-ray film and developed.



1 2 3 4 5 6 7 8

Figure 4. Comparison of recombinant rPLP-A protein with native rPLP-A. Equal amounts of protein from the conditioned medium of day 18 placental explants and rPLP-A transfected DG44 cells were separated by 12.5% SDS-PAGE, electrophoretically transferred and then immunoblotted with rPLP-A antipeptide antiserum.

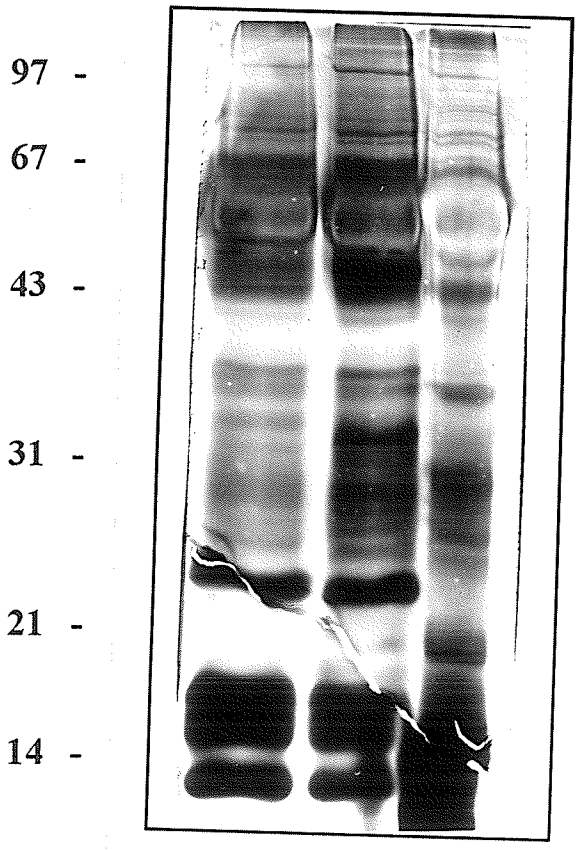


Placental Explant

Recombinant rPLP-A

Figure 5. Effect of tunicamycin treatment on *de novo* synthesis of rPLP-A from rPLP-A stably transfected CHO cells. Equal amounts of protein from rPLP-A transfected DG44 cells treated with tunicamycin (10 μg/ml) (lane 1) or without (lane 2), or from DG44 cells (lane 3) were separated by 12.5% SDS-PAGE, then either stained with silver (A) or electrophoretically transferred and then immunoblotted with rPLP-A antipeptide antiserum (B). There is not much difference in the amount of protein detected by silver-staining (A). In contrast, it is indicated by immunoblotting that the treatment of rPLP-A expressing cells with tunicamycin results in the identification of only a 25 kDa protein (arrow) rather than the 4 protein bands present in untreated cells (B).

A. Silver Staining



B. Immunoblotting

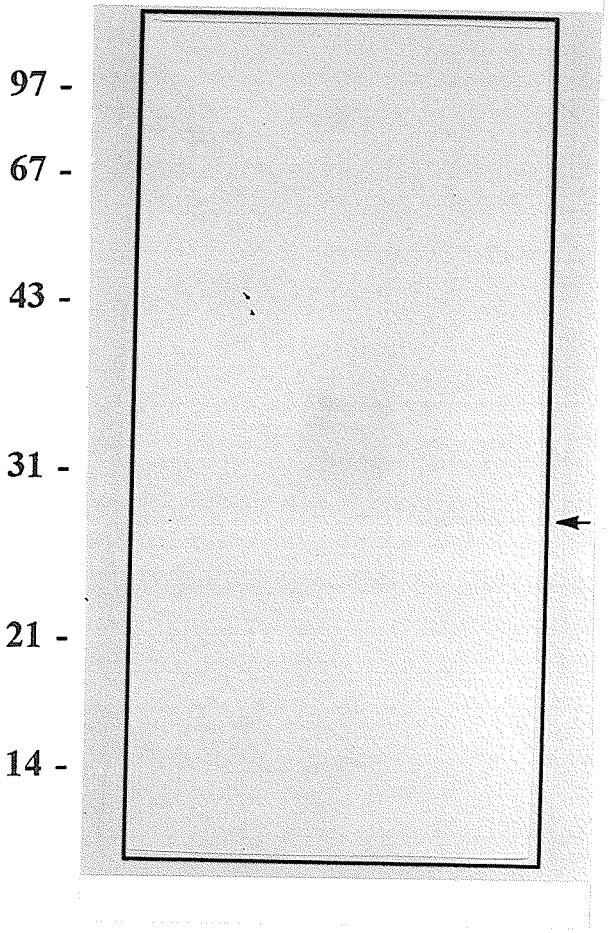


Figure 6. Affinity of recombinant rPLP-A for Concanavalin A and Wheat Germ Lectin. Equal amounts of protein from conditioned medium of rPLP-A transfected DG44 cells were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose. Immunoblotting was performed with rPLP-A antipeptide antiserum (lane 1) or protein blotting using biotin-labelled ConA (lane 2) or Wheat Germ Lectin (lane 3). Blots were then incubated with avidin-labelled horseradish peroxidase and color development followed as described by manufacturer.

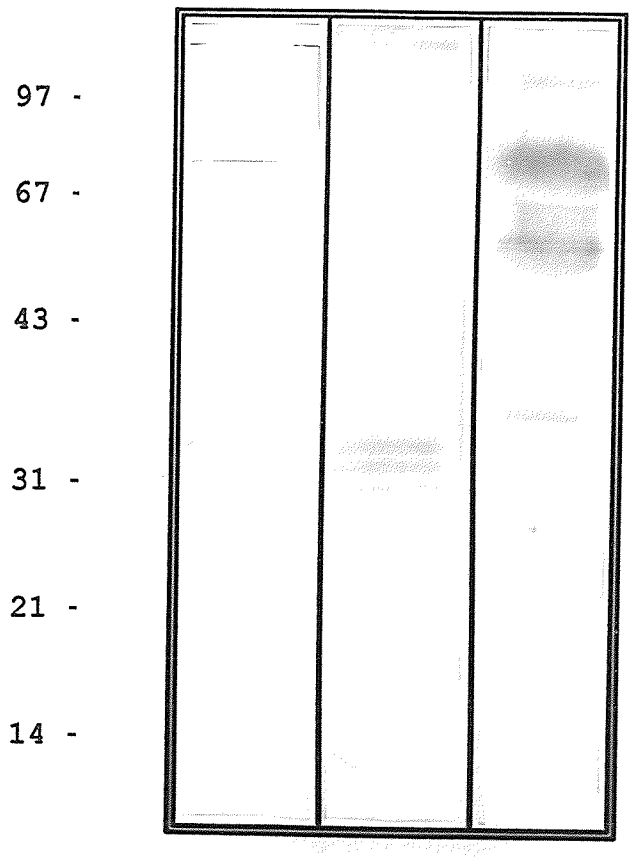

97 -
67 -
43 -
31 -
21 -
14 -
1
2
3
rPLP-A C.M.
rPLP-A C.M.
rPLP-A C.M.
Immunoblotting with Antibody
Protein blotting with ConA / Wheat Germ

Figure 7. Elution profile of recombinant rPLP-A from Sephadex G-100 column. 5 liters of conditioned medium from rPLP-A transfected DG44 cells was concentrated by precipitation in 100 mM $ZnSO_4$, the pellet was dissolved in 50 ml of 0.5 M EDTA, pH 8.0, containing 20 mM ß-mercaptoethanol, and then dialysed at 4°C against 50 mM HEPES, 100 mM NaCl, 10 mM EDTA and 20 mM ß-ME, 0.03% sodium azide, pH7.4 (Sephadex G-100 column buffer). The concentrated rPLP-A conditioned medium was then applied to a 1.2 L Sephadex G-100 column (5x120cm, equilibrated with Sephadex G-100 column buffer) and 12 ml fractions were collected. The fractions were monitored by absorbance at 280 nm.

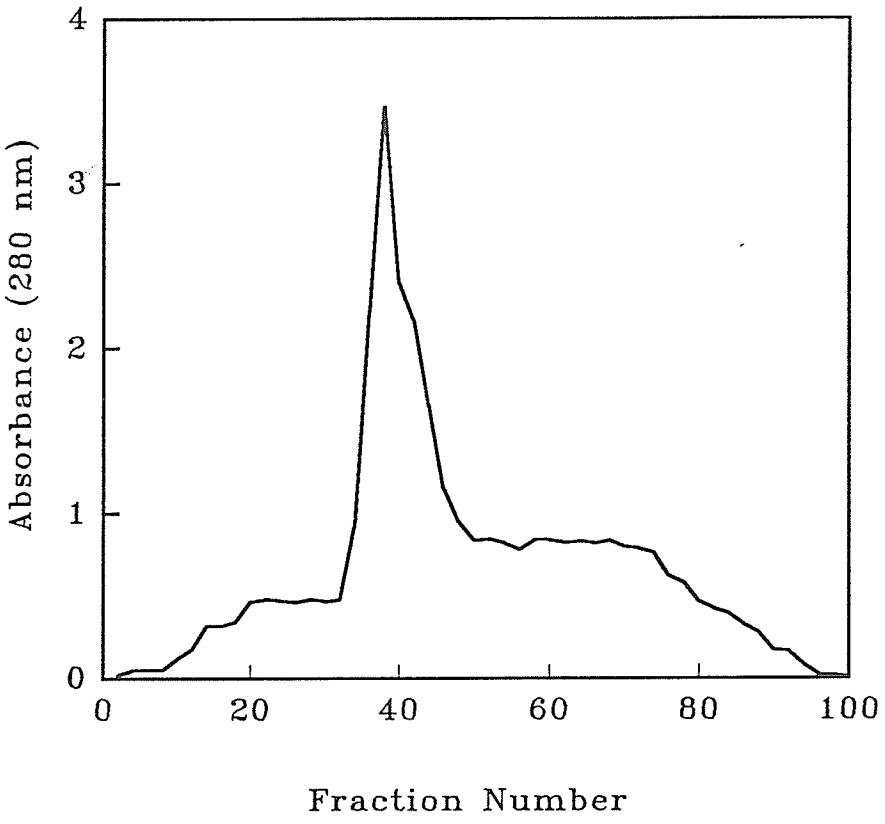
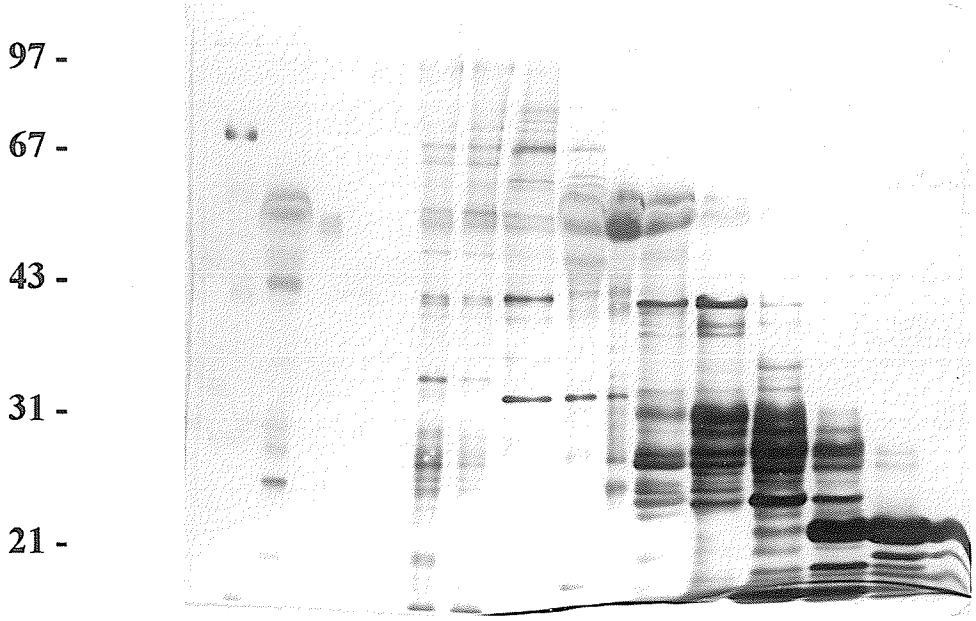

Absorbance (280 nm)
4
3
2
1
0
0
20
40
60
80
100
Fraction Number

Figure 8. SDS polyacrylamide gel electrophoretic pattern of Sephadex G-100 column fractions. Equal amounts of protein (starting from fraction 38 of Sephadex G-100 column and then every fifth fraction) were separated by SDS-PAGE, then either stained with silver (A) or electropheretically transferred and immunoblotted with rPLP-A antipeptide antiserum (B). Lane 1 is molecular weight marker, lane 2 is conditioned medium from rPLP-A transfected DG44 cells, lane 3 is conditioned medium from parental DG44 cells, lane 4 ia an empty lane, lane 5-15 are eluants from fraction number 38, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85, respectively.

A.


97 -
67 -
43 -
31 -
21 -
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B.

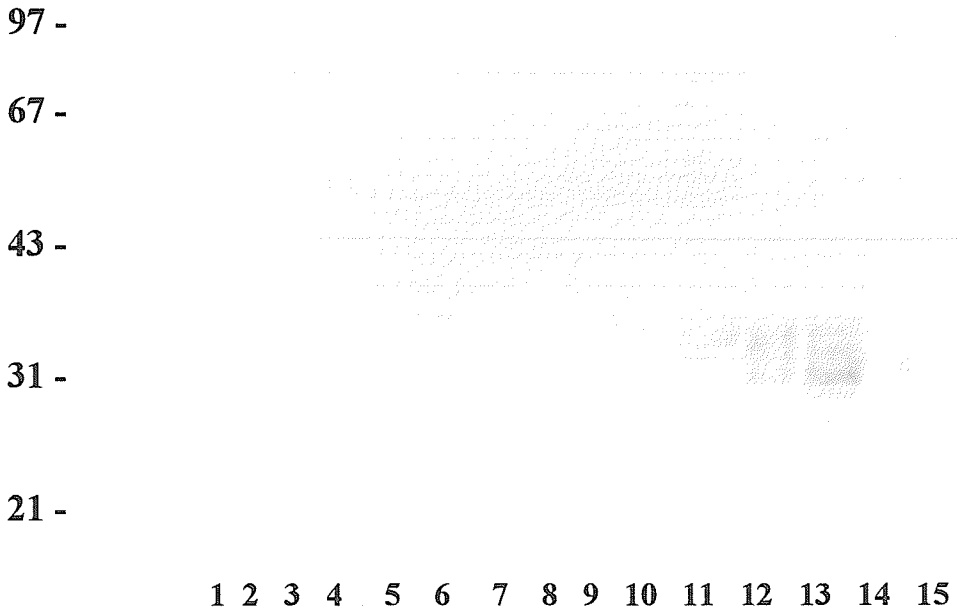

97 -
67 -
43 -
31 -
21 -
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 9. Elution profile of recombinant rPLP-A protein from a ConA affinity column. The rPLP-A immunoreactive fractions from a Sephadex G-100 column (fraction 65 to 85) were concentrated and dialysed extensively with 20 mM Tris-HCl buffer, pH 7.2, containing 150 mM NaCl, 10 mM EDTA, 2 mM ß-ME and 0.03% sodium azide (ConA column buffer). Samples was applied onto the ConA column (5x7cm) equilibrated with ConA column buffer. The column was washed with 4 bed volumes of column buffer and then absorbed proteins were eluted by 0.5 M α-methyl-D-mannopyranoside and 0.5 M α-methyl-D-glucopyranoside in ConA column buffer. Two ml fractions were collected and then monitored by absorbance at 280 nm.

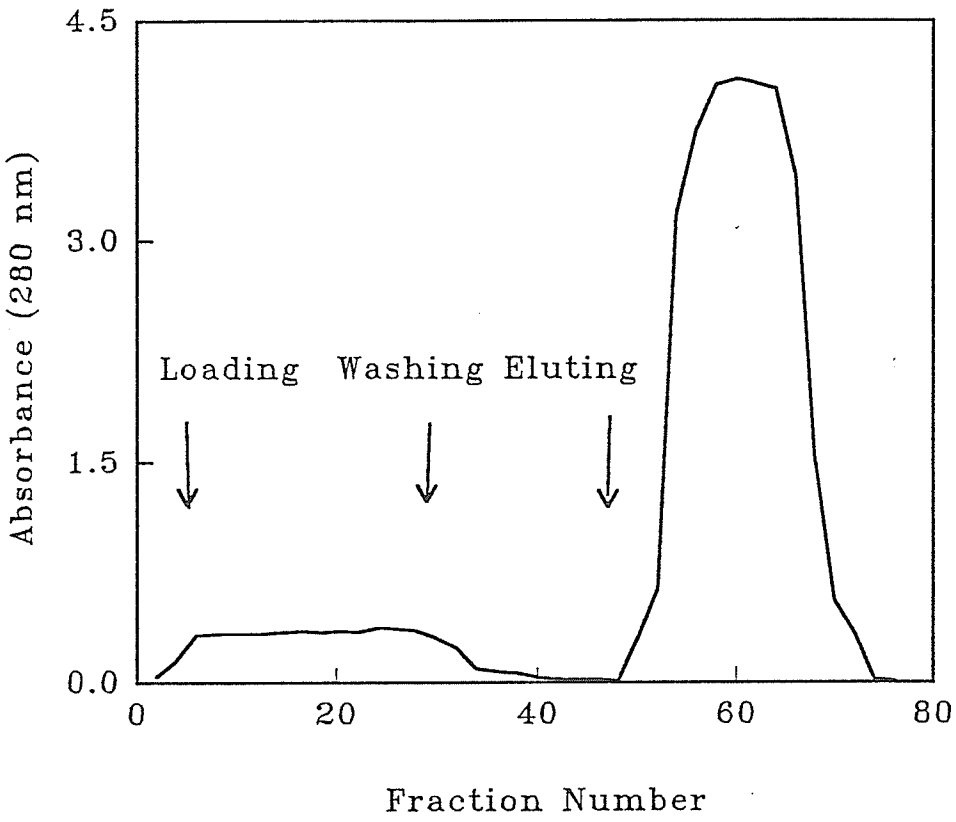
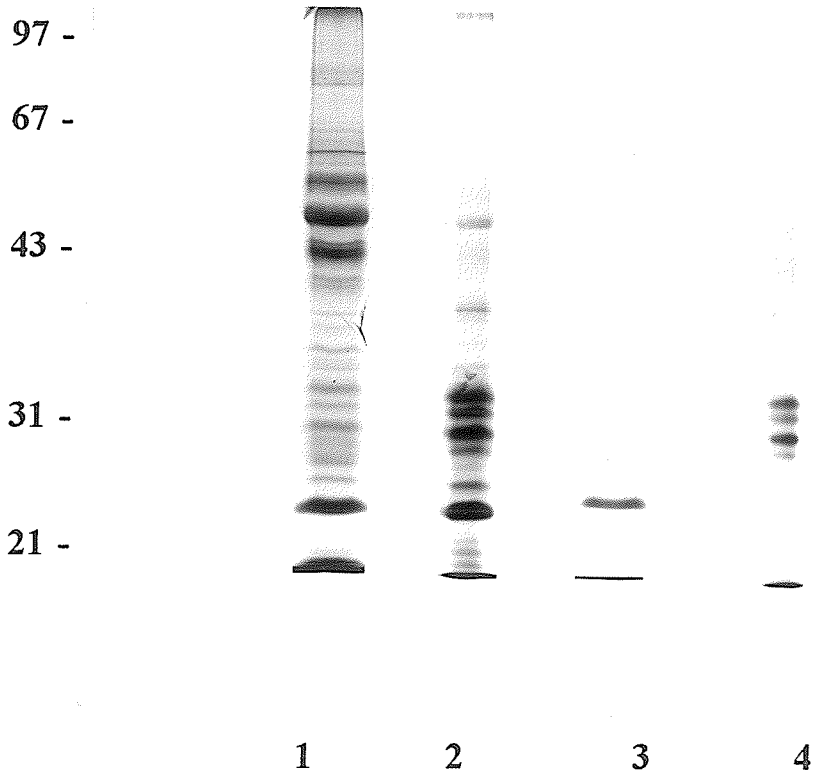

4.5
3.0
1.5
0.0
Absorbance (280 nm)
Loading
Washing
Eluting
0
20
40
60
80
Fraction Number

Figure 10. SDS-polyacrylamide gel electrophoretic pattern of recombinant rPLP-A following protein purification procedures. The concentrated conditioned medium from rPLP-A transfected DG44 cells (lane 1), pool from Sephadex G-100 column (lane 2), washing from ConA column (lane 3) and pool from ConA column (lane 4) were fractionated by SDS-PAGE and then stained with silver.



rPLP-A Pool from C. M. Sephadex ConA Column Washing Pool From ConA Column

Fig. 11. Displacement curves for rPLP-A peptide and purified recombinant rPLP-A protein in the RIA using iodinated rPLP-A peptide as the standard. Serial dilutions of rPLP-A peptide (solid circle), purified recombinant rPLP-A protein (open circle) as well as conditioned medium from DG44 cells (solid triangle) were measured in the double antibody radioimmunoassay. The concentration of rPLP-A protein and peptide were converted to nM (molarity was calculated according to mature rPLP-A protein and peptide #129-145) in the figure for easy of comparison. Each point represents the mean of the duplicates.

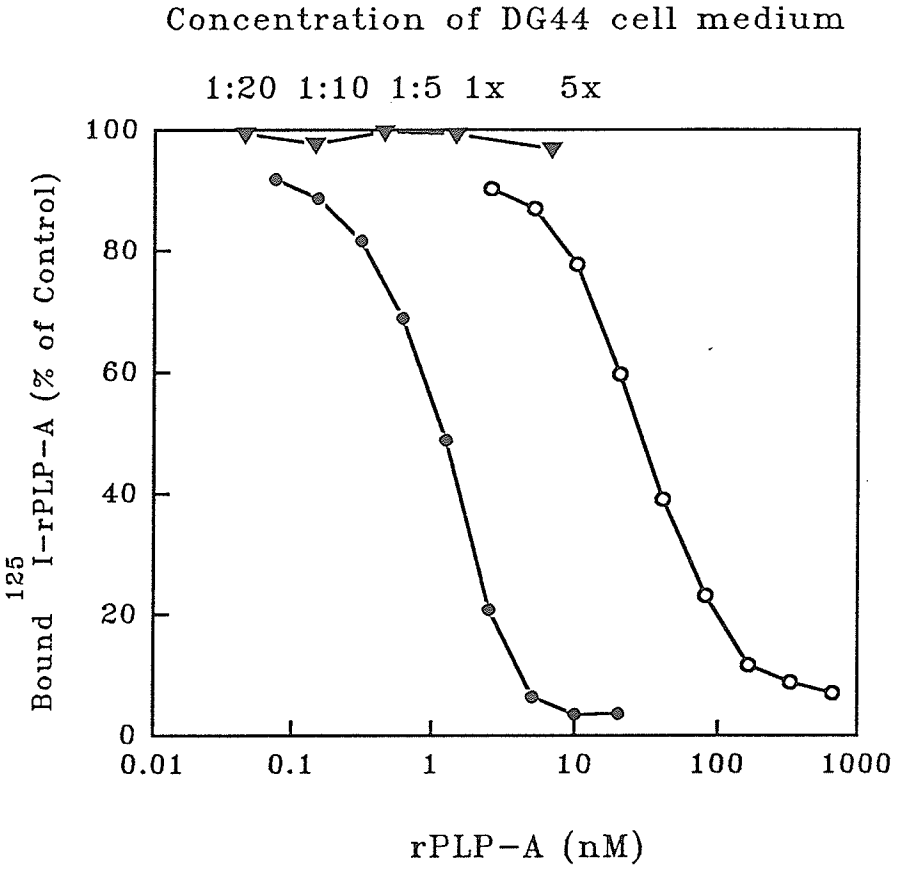

Concentration of DG44 cell medium
1:20 1:10 1:5 1x 5x
100
80
60
40
20
0
Bound 125I-rPLP-A (% of Control)
0.01
0.1
1
10
100
1000
rPLP-A (nM)

Fig. 12. Displacement curves for the rPLP-A peptide, serum, placental extract and amniotic fluid from day 20 pregnant rats. Serial dilution of rPLP-A peptide (solid circle), serum (open circle), placental extract (solid triangle), placental explant (solid square) and amniotic fluid (open square) from day 20 pregnant rats were measured in the double antibody radioimmunoassay. The rPLP-A peptide was used as standard. Each point shows the mean of duplicates.

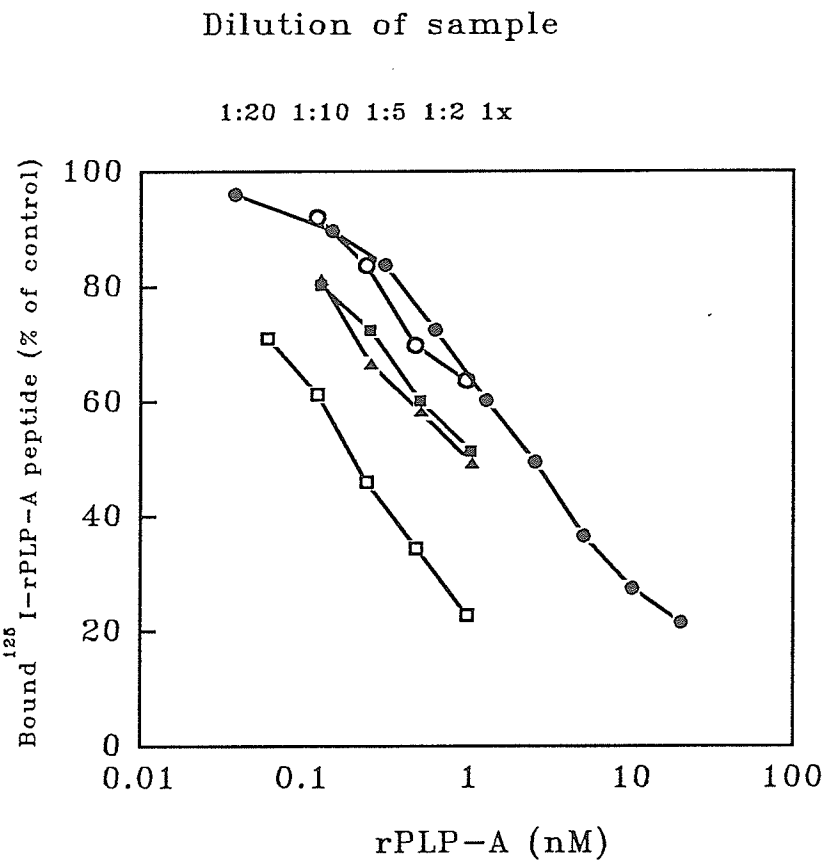

Dilution of sample
1:20 1:10 1:5 1:2 1x
Bound ^{125}I-rPLP-A peptide (% of control)
100
80
60
40
20
0
0.01
0.1
1
10
100
rPLP-A (nM)

Fig. 13. Gestational profile of the rPLP-A concentrations in maternal serum and day 20 amniotic fluid as measured by the RIA. Each point represents the mean and standard error (SE) of values from four rats. Maternal serum (open bars) and amniotic fluid (hatched bars) were assayed in duplicates. The purified recombinant rPLP-A protein was used as a standard.

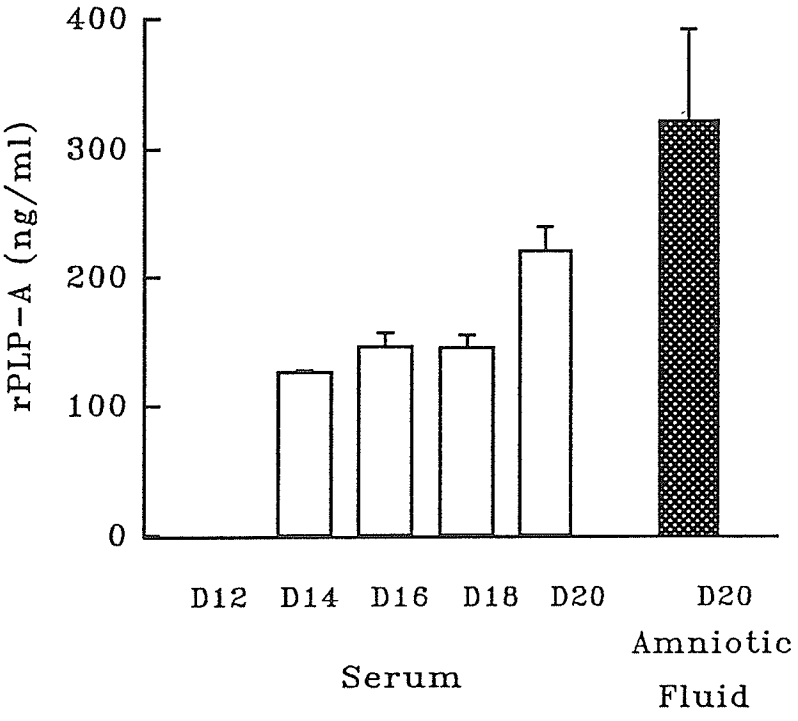
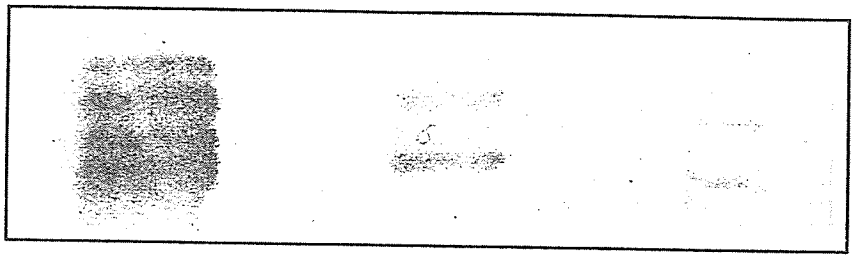

400
300
200
100
0
rPLP-A (ng/ml)
D12
D14
D16
D18
D20
D20
Amniotic
Fluid
Serum

Fig. 14. Immunoblotting of amniotic fluid collected from day 20 pregnant rats using rPLP-A antipeptide antiserum. The recombinant rPLP-A (lane 1), placental extract (lane 2) and amniotic fluid from day 20 pregnant rats were fractionated by 12.5% SDS-PAGE, electrophoretically transferred to nitrocellulose membrane and immunoblotted with rPLP-A antipeptide antiserum.



Recombinant rPLP-A

Placental Extract

Amniotic Fluid

Fig. 15. Effects of ovariectomy (Ovx) and/or adrenalectomy (Adx) on serum levels of rPLP-A. Pregnant rats were subjected to Ovx (solid bars), Adx (open bars), Ovx plus Adx (hatched bars), or sham operations (crossed bars) at day 16 of gestation. Blood was collected on subsequent days. Serum rPLP-A level were significantly different between Ovx/Adx and sham group at day 17, 18, 19, 20, 21 as well as between Ovx and sham group at day 18, 19, 20 and 21. Each bar represents the mean and standard error (vertical line) of values from 3 rats.

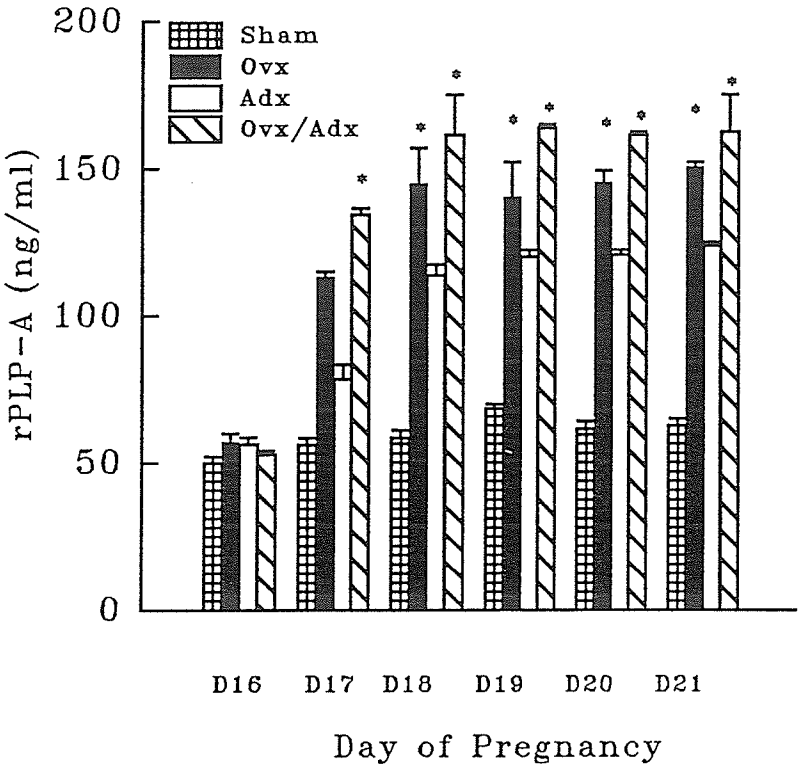

200
150
100
50
0
rPLP-A (ng/ml)
Sham
Ovx
Adx
Ovx/Adx
D16
D17
D18
D19
D20
D21
Day of Pregnancy

Fig. 16. Effects of ovariectomy (Ovx) and/or adrenalectomy (Adx) on serum level of rPL-II. Pregnant rats were subjected to Ovx (solid bars), Adx (open bars), Ovx plus Adx (hatched bars), or sham operations (crossed bars) at day 16 of gestation. Blood was collected on subsequent days. Serum rPL-II level were significantly different between Ovx/Adx and sham group at day 17, 18, 19, 20, 21 as well as between Ovx and sham group at day 20 and 21. Each bar represents the mean and standard error (vertical line) of values from 3 rats.

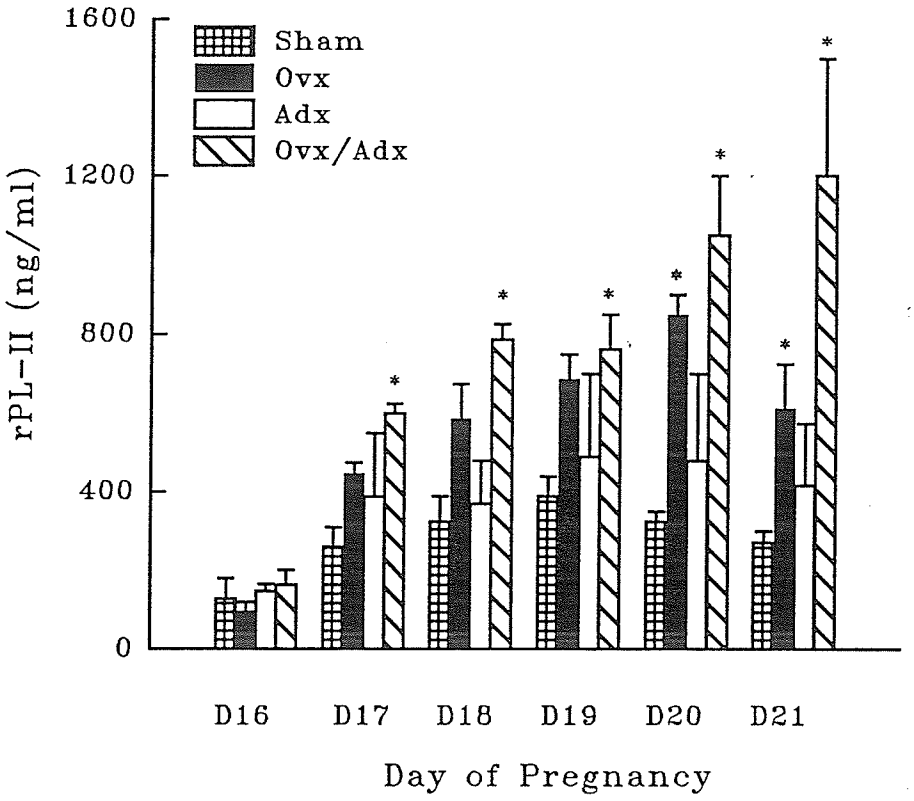
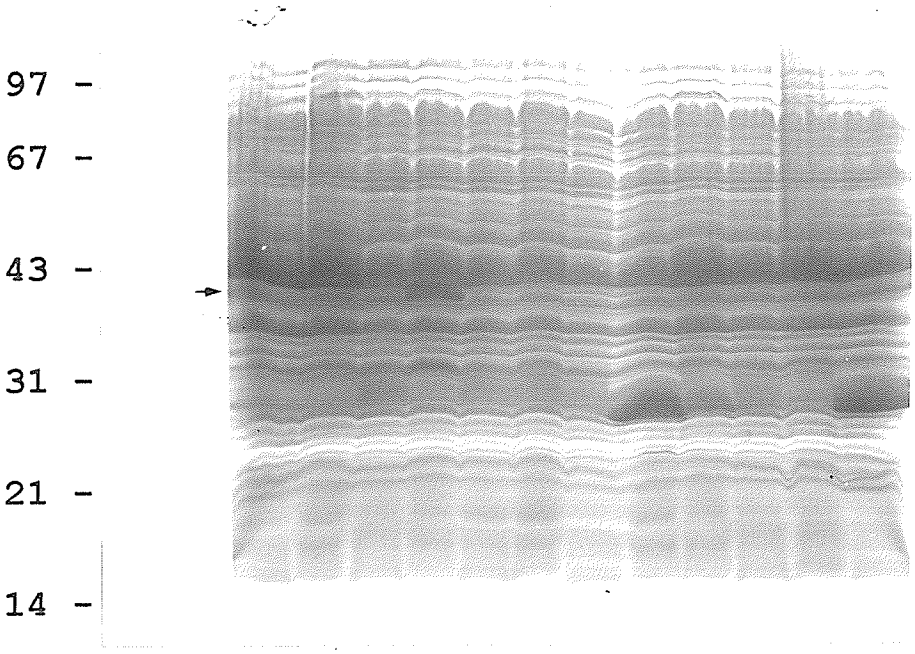

1600
1200
800
400
0
rPL-II (ng/ml)
Sham
Ovx
Adx
Ovx/Adx
*
D16
D17
D18
D19
D20
D21
Day of Pregnancy

Figure 17. Screening by SDS-PAGE for clones containing a rPLP-A/GST fusion protein. The individual clones of the rPLP-A/GST transformants were grown in 5 ml of LB medium containing 100 μg/ml of ampicillin in the presence of 0.1 mM of IPTG for 5 hr, cells were collected and resuspended in 100 μl of SDS-PAGE loading buffer and then 20 μl were separated by 10% SDS-PAGE for the screening of the expression of rPLP-A/GST fusion protein using Coomassie blue R-250 staining. (The expected size for rPLP-A/GST fusion protein is 43 kDa while GST alone is 26 kDa).

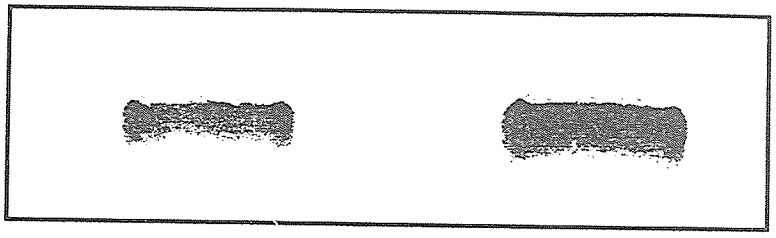


1 2 3 4 5 6 7 8 9 10 11 12

Vector 18 19 20 21 22 23 25 26 27 28

1 2 rPLP-A/GST fusion clones

Figure 18. Purification of rPLP-A/GST fusion protein. The fusion protein positive colony (No. 19) was grown in 1 L of LB medium containing 100 μg/ml of ampicillin in the presence of 0.1 mM IPTG for 5 hr. The cells were collected and then resuspended in 2 ml of SDS-PAGE loading buffer, boiled at 100°C for 5 min and then loaded onto 10% SDS gel. The rPLP-A/GST fusion protein band (43 kDa protein) was cut from the gel, electroeluted and electrodialysed with NH_4HCO_3 and SDS. The purified protein was dialysed and concentrated by ultrafiltration (Amicon UM 10) and 5 μl and 10 μl of purified fusion protein (concentration: 1 mg/ml) were separated by SDS-PAGE, then stained with Coomassie Blue R-250.



GST--rPLP-A fusion protein

5 ul 10 ul