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Roles of the mitogen-induced proteins, mitogen regulated protein/proliferin and 24p3/uterocalin, during mouse development

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Roles of the mitogen-induced proteins, mitogen regulated protein/proliferin and 24p3/uterocalin, during mouse development.

by

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A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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For the Major Program
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ABSTRACT

Mitogens, serum and growth factors, induce the secretion of several proteins in cell cultures. Mitogen regulated protein/proliferin (MRP/PLF) is one such protein secreted from 3T3 cells 20 hours after the addition of fibroblast growth factor (FGF). 24p3/uterocalin is another protein induced 10 hours after FGF addition. When combined with cycloheximide, 24p3/uterocalin secretion is increased by 5 fold.

MRP/PLFs are glycoproteins, belonging to the prolactin-growth hormone family, expressed at high concentration during mid-gestation in the mouse placenta. MRP/PLFs are thought to stimulate angiogenesis in the placenta and the uterus, and stimulate uterine cell growth during mid-gestation. To further study the roles of these glycoproteins, recombinant MRP/PLFs were created, and expressed and purified in 293 human fetal kidney cells.

To provide insights into the potential regulation of uterine cell proliferation by MRP/PLFs, the profiles of the wet weight and DNA content of the uterus were characterized and found to have a positive correlation with the expression profile of MRP/PLFs. Direct examination of MRP/PLF stimulation by estradiol-17β or progesterone in day 8 and 9 placental minced, organ cultures demonstrated no effect on secreted MRP/PLF levels. Purified MRP/PLFs were used to further study the uterine MRP/PLF receptor on primary uterine cell and organ cultures, and in an in vitro day 11 uterine membrane binding assay. No evidence of biological or binding activity to the uterine MRP/PLF receptor was detected.

Expression of 24p3/uterocalin was detected in the embryonic spine on gestational day 13 and shown to increase throughout gestation and over the life time of the mouse. In the
liver 24p3/uterocalin mRNA levels were the highest in the late-gestational fetal liver and decreased within the first week after birth. The 24p3/uterocalin mRNA was also detected in the spleen of juvenile adults. The presence of 24p3/uterocalin mRNA in the fetal and juvenile liver and in the spine after birth appeared to correlate with hematopoiesis in these tissues and therefore it is hypothesized that 24p3/uterocalin may have a potential role in events related to both embryonic and adult hematopoiesis.
CHAPTER 1.

GENERAL INTRODUCTION

DISSERTATION ORGANIZATION:

This dissertation contains five chapters. Chapter 1 is a literature review of mouse gestation accompanied by a description of the biochemical and functional properties of the prolactin-growth hormone family with an emphasis on one of its member; the growth factor regulated mitogen regulated protein/proliferin (MRP/PLF). Chapter 2 describes the research accomplished to express and purify glycosylated recombinant MRP/PLFs from mammalian cells. Chapter 3 describes the characterization of the MRP/PLF receptors and a search to find novel roles for MRP/PLFs. Chapter 4 describes the characterization of another growth factor regulated protein called 24p3/uterocalin by showing its expression pattern in embryonic, juvenile and adult mouse. Chapter 5 contains a general discussion on the research findings as well as recommendations for future studies. Finally, an appendix is included to describe previous experiments aimed at developing tools to characterize MRP/PLFs interactions with known receptors.

INTRODUCTION:

During the course of evolution, mammals distinguished themselves from other animals by devising a new strategy for the development and the protection of their embryos. The embryos develop inside the uterus and physiological communication with the mother is established by the placenta. This temporary organ of embryonic origin connects the embryo to
the uterus and allows efficient exchange of nutrients and wastes between the mother and the embryo. Prior to the formation of the placenta, biochemical signals allow the first critical step of embryo development which is its implantation in the uterus.

In mice, the gestational period is approximately 20 days beginning with fertilization of the oocyte at day 0 and implantation of the embryos (usually 10 to 12) at 4.5 days. By mid-gestation, organogenesis of the embryo is virtually complete and the remaining half of the gestational period enables the embryo to quadruple in size. During mid-gestation there is a switch in the type of physiological communication occurring between the mother and the fetus. Prior to mid-gestation, most of the gas and nutrient exchanges have occurred through the yolk sac membrane. However, during the second half of gestation, the newly formed placenta becomes the critical organ in the biochemical communication between the mother and the growing fetus. The final stage in the relationship between the mother and the fetus involves the preparation for parturition (1).

The prolactin-growth hormone (PRL-GH) family contains several placental proteins secreted by the placenta during gestation. The specific subfamily of mitogen regulated protein/proliferins (MRP/PLFs) are expressed during mid-gestation in the mouse. However, the role played by MRP/PLFs during this critical period in mouse development is not clear. Mid-gestation is the most critical period in mouse development because failure to establish proper communications with the uterus will result in rapid resorption of the embryo.
SUMMARY OF MOUSE GESTATION:

*Development of the fertilized egg to the preimplantation blastocyst*

After fertilization of the oocyte in the oviduct (day 0.5), the egg progressively starts DNA synthesis and cell division. Until the first cell division, the embryo relies primarily on the maternal proteins and RNAs accumulated during oogenesis (1). By the middle of the two cell stage (day 1.5), the embryonic genes are switched on (2) and degradation of most maternal RNAs and proteins increases (3, 4). Until uterine implantation, development of the embryo does not seem to require exogenous growth factors as demonstrated by *in vitro* culture of embryos (5, 6).

As the cells divide, the embryo will undergo three morphogenetic transitions prior to implantation (Figure 1). The first transition known as compaction begins at the eight-cell stage (day 2.5) when cells flatten and start forming tight junctions and gap junctions. The first embryonic “epithelium” is formed when the cells in contact with the uterine lumen evolve into the trophectoderm (TE). This “epithelium” encloses the first embryonic “endoderm” when the cells having no contact with the uterine lumen evolve into the inner cell mass (ICM) (7).

The 32-cell (day 3) embryo undergoes the second morphogenetic transition called cavitation. During this process, the blastocoel, a cavity filled with liquid, is formed by the action of Na/K-ATPase pumps present on the basolateral membranes of the TE (8). At this stage, the TE begins to differentiate into the polar TE, cells in contact with the ICM, and the mural TE which encloses the blastocoel and does not contact the ICM (7).

During the third morphogenetic transition, blastocoel expansion occurs with the continued activity of the Na/K-ATPase pumps. Expansion and contraction of the blastocyst,
together with enzymatic digestion, triggers its release from the zona pellucida (day 4).

Arrangement of the blastocysts in the mouse uterus is determined by uterine contractions and the implantation of blastocysts will only occur if the uterus is primed by reproductive steroids (9).

Figure 1: Schematic of the major changes during the development of the fertilized egg. After fertilization the embryo undergoes compaction at the 8 cell stage. After further cell division the embryo undergoes cavitation when the blastocoel is formed. During cavitation, the cells keep on dividing and the embryo is now called a blastocyst. After blastocoel expansion, the blastocyst implants to the uterine epithelium. TE: Trophectoderm, B: Blastocoel, ICM: Inner cell mass, UE: uterine epithelium.
Preparation of the uterus for implantation

The uterus is prepared for implantation of the blastocysts by the action of ovarian estradiol and progesterone. This initial phase of the uterine preparation is independent of the presence of blastocysts in the uterine lumen. During ovulation (day 0.5) in the normal murine estrus cycle, estradiol-17β is released by the ovaries and stimulates cell proliferation of the uterine luminal epithelium. Progesterone, secreted by the ovaries, normally rises and falls four days after ovulation, the length of a single estrus cycle. Mating will cause the extension of progesterone secretion to 9 days via bi-daily pituitary pulses of prolactin. Progesterone secretion initiates a switch in the luminal epithelial cells from the actively proliferating phenotype to a differentiated secretory phenotype while simultaneously stimulating stromal cell proliferation. An “implantational pulse” of estrogen secreted by the ovaries (day 3) will then further stimulate growth of the uterine stromal cell population until the uterine lumen is forced to close around the blastocyst.

Implantation of the blastocyst into the uterus

Implantation requires the contact of the blastocysts with the uterine epithelium (day 4.5) followed by attachment and invasion of the blastocysts into the uterine stroma. The ability of the blastocysts to successfully contact the uterine epithelium is governed by the release of estrogen from the ovaries during the “implantational pulse”. This estrogen pulse stimulates apoptosis of the uterine luminal epithelial cells in contact with the mural TE. Death of the uterine epithelial cells, which are phagocytosed by the trophoblastic giant cells, allows the embryonic tissue to “invade” the endometrium (16, 17) (Figure 2). Finally,
“invasion” of the embryo causes the uterus to respond by initiating decidualization (day 5.5), a process intrinsic to the uterus and required for implantation as described in the following section.

Figure 2: Schematic representation of a cross-sectioned gestational uterus at day 9. Once implanted the embryo is surrounded by several differentiated cell types: UE: uterine epithelium; PGC: primary trophoblastic giant cells; SGC: secondary trophoblastic giant cells; UC: uterine crypt; EMB: embryo.

Transformation of the uterus after implantation

Decidualization refers to the further increase in uterine size and weight after implantation of the blastocyst. The uterine stroma continues its rapid growth by cell multiplication, followed by differentiation, leukocytes infiltration (18, 19) and edematous swelling of the uterine tissue due to a change in the vascular permeability (20). These changes ultimately lead to the formation of a new tissue: the decidua. However, the myometrium,
outer section of the uterus, is not modified and remains for the reconstruction of the uterine stroma and luminal epithelium after parturition (17). The uterus will be fully decidualized by day 14. However by day 9, the decidua in contact with the slowly growing embryo begins to undergo slow regression until day 14. From day 14 to parturition, the rate of regression increases dramatically to accommodate the rapidly growing embryo. The decidual regression continues until the embryo reaches the myometrium of the uterus (9, 21).

The decidua is thought to play several roles in gestation. The newly decidualized uterine cells form a tight barrier around the embryo to protect it from the maternal immune system by restricting the passage of immunoglobulins, lymphocytes and even microorganisms (22). The decidua, in turn, protects the mother by moderating the “invasiveness” of the embryo by secreting several protease inhibitor, like TIMP-1, TIMP-3, for the proteases B/MMP9, uPA, secreted by the embryonic trophoblast (15).

Formation of the placenta

After implantation (day 5), the mural and polar trophectoderm will undergo different fates. The mural trophectoderm cells stop dividing and undergo enderoreplication, DNA replication without cell division, giving rise to the primary trophoblastic giant cells (23). The polar trophectoderm cells continue to proliferate and migrate in several directions. As the cells migrate away from the ICM they eventually stop dividing and differentiate into three major cell types. First, some polar TE cells migrate around the embryo and undergo enderoreplication of their DNA to replace some of the primary trophoblastic giant cells. Second, some TE cells migrate into the blastocoel cavity to ultimately become a part of the
umbilical cord. Third, some TE cells will migrate further in the endometrium and anchor the
embryo in the uterus. This third TE cell population differentiates to form a part of the placenta
and the TE cells in contact with the endometrium will evolve into secondary trophoblastic
giant cells by enderoreplication of their DNA (1, 24).

Transport of nutrients from the mother to the fetus occurs between day 9 and day 13
through the “yolk sac” placenta. This primitive placenta is formed by the primary
trophoblastic giant cells and cells originating from the embryo. The primary trophoblastic
giant cells “invade” the decidua to come in contact with the maternal circulation and initiate
the formation of blood sinuses. These trophoblastic giant cells enable nutrients, gas and waste
to be exchanged between the mother and the embryo (15).

At approximately day 10, exchanges of nutrient and waste between mother and embryo
begin to shift from the “yolk sac placenta” to the true placenta. The bulk of the placenta is
made from cells derived from the ICM and the polar trophectoderm. The available secondary
trophoblastic giant cells will connect the embryo to the maternal circulation via blood sinuses
(15).

The placenta plays an important role in the maintenance of pregnancy (24). It allows
the exchange of nutrients that are necessary for the successful development of the fetus.
Through the placenta, the fetus communicates with the mother to keep the hormonal balance
in a “pregnancy state” and to prepare the mother for lactation. In particular, the placenta
synthesizes several members of the prolactin-growth hormone family which allows for the
maintenance of gestation and preparation for lactation (25, 26).
THE RODENT PROLACTIN-GROWTH HORMONE FAMILY:

Interestingly, although the prolactin-growth hormone family constitutes part of the major proteins secreted from the mouse placenta and the decidua during gestation, the first two members of this family, prolactin (PRL) and growth hormone (GH) were initially cloned from murine anterior pituitary gland (27). In the mouse, prolactin is released from the anterior pituitary throughout gestation to induce the corpus luteum's secretion of progesterone to maintain pregnancy (luteotropic action). However, it was shown that when the anterior pituitary is removed after mid-gestation, pregnancy can be maintained without prolactin suggesting another source for a PRL-like activity. This activity was identified in placental extracts (28). Subsequently, isolation of the proteins which had this PRL-like activity and subsequent cloning of their cDNAs identified placental lactogen I and II, PL-I and PL-II respectively, as members of the PRL-GH family (29, 30). Several additional members were discovered by finding sequence homologies in Expressed Sequence Tag databases (31-38). To date, over 20 members comprising the PRL-GH family have been characterized.

A common feature for this family of proteins lies in their tissue specific expression patterns. All family members, except for PRL and GH, which are secreted by the anterior pituitary, are produced during gestation by the placenta or the decidua. The trophoblastic giant cells of the placenta are known to express all the currently characterized members of the PRL-GH family (25).

A second feature is a common exon/intron structure suggesting that all the members of the mouse PRL-GH family arose from gene duplication of prolactin and are all located on chromosome 13. The typical gene structure is defined by five exons and four introns.
However, members of one subfamily, the prolactin like protein-C (PLP-C), have an additional small exon coding for an aromatic amino acid rich region between exons two and three. The significance of this extra intron is still unknown but it is believed to modulate the PLP-C receptor binding activities \textit{in vivo} (33, 36, 38, 39).

A third common characteristic of PRL-GH family members lies within the conserved features of their protein primary structure. All family members are secreted proteins of approximately 200 amino acids and can be classified according to the number of conserved cysteines. Each individual member contains at least four conserved cysteines (three of them located at the C-terminus) which are involved in the formation of intramolecular disulfide bridges. Some family members contain an additional cysteine in the middle of the protein or two additional cysteines at the N-terminus. With the exception of PL-II, all family members are post-translationally modified by the addition of carbohydrates. The structure and extent of the glycosylation depends on the time during gestation and cell type expressing these proteins. The variation in glycosylation add a new level of regulation for the action of PRL-GH family members (25). For example, the degree of glycosylation of PRL affects the activity of human pituitary prolactin in the pigeon crop assay (40) and rat lymphoma Nb2 cell based prolactin receptor binding assay (41).

Despite their common features, there has been great difficulty in defining all the biological functions and receptors for the PRL-GH family members. The “classical members” such as PRL, GH, PL-I and PL-II are the best characterized and bind to the PRL receptor to play roles in gestation as mammotrophic, stimulation of mammary gland development, and luteotrophic hormones. These same PRL-GH family members also play over 300 different roles
in cell proliferation and differentiation, osmoregulation, metabolism, behavior, reproduction and maintenance of optimum immune protection at the cellular level (42). The cellular and molecular mechanisms of these roles were better characterized using mouse PRL receptor knock-outs (43). The biological functions and receptors for the “non-classical” members are gradually being defined. One of the best characterized PRL-GH “non-classical” subgroups is the family of mitogen regulated protein/proliferin (MRP/PLFs).

CHARACTERIZATION OF MRP/PLFS AND THEIR RECEPTORS:

*Discovery of MRP/PLF and their gene structure*

Mitogen regulated proteins (MRPs) are 34 kDa proteins and were first discovered as a family of glycoproteins secreted by mice Swiss 3T3 cells and Balb/c normal liver cells (BN/L) stimulated by epidermal growth factor (EGF) and fibroblast growth factor (FGF) (44). Proliferin (PLF) was originally identified, by differential screening of a cDNA library, as a 422 bp mRNA transcript upregulated in response to serum stimulation of quiescent Balb/c 3T3 cells (45). The deduced amino acid sequence from the mRNA revealed that PLF belonged to the prolactin/growth hormone family (46). Cloning of the MRP cDNA from BN/L cells showed that MRP was identical to PLF (47).

Further characterization of *mrp/plf* by Southern blot analysis suggested that there are at least four to five copies of the *mrp/plf* gene present in the mouse genome (48). There are four characterized *mrp/plfs: plf1* (46), *plf2* (49), *mrp3* (50) and *mrp4* (51). *Plf1, plf2, mrp3* are 99 % identical at the cDNA level, differing by only three base pairs. However, *mrp4* (cloned from day 13 placental RNA) is 97 % identical to *plf1* (51).
MRP3, the only member of the MRP/PLF family with a known gene structure, possesses the characteristic five exons/four introns structure of the prolactin/growth hormone family members (25, 50). MRP/PLFs have been mapped to chromosome 13 (39). The deduced amino acid sequences show three N-glycosylation consensus sequences for PLF1, PLF2 and MRP3, and only one for MRP4 due to base pair changes (Figure 3). During mid-

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Figure 3: Amino acid alignment for MRP/PLFs and mouse prolactin.
The amino acid sequences deduced from the cDNA for mouse prolactin (mPRL) and MRP/PLF (PLF1, PLF2, MRP3, MRP4) were aligned according to the position of the 6 conserved cysteines (indicated by the I above and underneath the sequences). The amino acids of the secreted proteins are in capital letters and the amino acid from the signal sequences are in lower case and italicized. The putative recognition sequences for N-glycosylation (NXS motif) are underlined within the sequences. The conserved amino acids of mPRL and all 4 MRP/PLFs are represented by the * above the sequences. The conserved amino acids within the MRP/PLF family are represented by the ^ underneath the sequences.
```
gestation *mrp/plf* mRNAs and proteins were detected by *in situ* hybridization and immunohistochemistry in trophoblastic giant cells (52). These characteristics confirmed that MRP/PLFs were true members of the prolactin/growth hormone family.

**Receptors for MRP/PLFs**

Recombinant PLF1 expressed and purified from CHO (Chinese hamster ovary) cells was shown to bind to the IGFII/mannose-6-phosphate /cation independent (IGFII/M6P) receptor from the mouse placenta and the liver of both fetuses and pregnant mice. The maximal binding activity was observed in day 16 maternal and fetal liver membranes. Placental membrane binding was shown to increase from the formation of the placenta (day 10 of gestation) throughout the end of pregnancy (day 19 to 20) (53).

Another, as yet to be identified, receptor was later characterized in day 11 uterine membranes. MRP/PLFs isolated from the conditioned medium of FGF stimulated BN/L cells were shown to bind to uterine membranes with the maximal binding activity observed in day 11 uterine membranes. Excess cold mannose-6-phosphate was used in competitive binding experiments to establish that the characterized binding activity was not to the IGFII/M6P receptor but rather to the putative uterine receptor. Furthermore, PRL, PL-I or GH do not compete with MRP/PLFs for binding to the uterine membranes suggesting that the characterized receptor is not the PRL receptor (54). These results supported the observation that recombinant PLF1 did not bind to the prolactin or growth-hormone receptors (53) and led to the recognition of MRP/PLFs as “non-classical” members of the PRL/GH family (25).
Detection of MRP/PLFs binding in vivo

By radioimmunoassay, MRP/PLFs were found to be secreted into the maternal bloodstream and the amniotic fluid with a peak of expression during mid-gestation (52). Iodine-labeled PLF1 bound to embryonic heart and blood vessels in the area of the dorsal artery between days 10 and 18 of gestation. Also, at the same days of gestation, immunohistochemistry studies detected MRP/PLFs in the endothelial cell lining of blood vessels of the developing vertebrae and ribs of the embryo (55).

Interestingly, temporal expression patterns during gestation differ between MRP/PLF family members within the placenta. The profile of expression of the different mrp/plfs in the placenta was established by RT-PCR followed by specific restriction enzyme digestion which allowed for a differentiation between all the known mrp/plfs at the DNA level. These profiles showed that mrp3 constituted the majority of the mrp/plfs present in the placenta. Plfl was also present to a lesser extent and matched the profile of mrp3 expression. However, mrp4 was expressed later during gestation with a peak at day 12 compared to a peak at day 10 for mrp3 and plfl. A smaller 27 kDa form of MRP/PLF can be detected by Western blot in addition to the 34 kDa form. The secretion of the 27 kDa protein during gestation matches the profile of expression of the mrp4 mRNA. (56).

MRP/PLFs were the first members of the PRL-GH family aside from PRL and GH that are found to be expressed in both male and female adult mice beyond gestation. For instance, mrp4 mRNA was detected in the adult tail and ear by RT-PCR. Immunohistochemical studies localized the expression of MRP4 to the keratinocytes of the outer root sheath of the tail hair follicles (51). MRP3 was detected in the adult mouse during
wound healing in keratinocytes near the wound edge. In primary newborn keratinocyte cell cultures, \textit{mrp}3 mRNA is upregulated by keratinocyte growth factor. During hair follicle morphogenesis, MRP3 is expressed in the outer root sheath during mid-anagen with the highest expression in late-anagen which might suggest a role for MRP3 in hair follicle cycling (57).

\textbf{Physiological functions of MRP/PLFs}

The binding of recombinant PLF1 to the endothelial cell lining of blood vessels suggested a possible role in angiogenesis. Recombinant PLF1 was shown to induce the migration of bovine capillary endothelial cells, and stimulate neovascularization in the rat cornea assay (58). The addition of mannose-6-phosphate inhibited the angiogenic activity of PLF1 in the endothelial cell migration assay suggesting that the IGFII/M6P receptor was required for this activity (59). Further characterizing of the angiogenic activity showed that PLF1 can induce endothelial cell migration through a G-protein coupled MAPK-dependent pathway (60).

Also, in the GATA 2 and 3 transcription factor knockouts, MRP/PLFs protein and mRNA are down-regulated in the placenta which correlate with the decrease in blood vessel formation in the placenta and the uterus. The \textit{mrp/plf} promoters were shown to contain the GATA 2 and GATA 3 consensus binding sites for the GATA transcription factors. Therefore it has been hypothesized that expression of MRP/PLFs in the placenta during mid-gestation is regulated by GATA 2 and GATA 3 and that MRP/PLFs are involved in the formation of blood vessels in the decidua (61). Furthermore, MRP/PLFs stimulate thymidine incorporation
in mid-gestational primary uterine cell cultures suggesting a role for MRP/PLF in uterine cell proliferation (54).

These results suggest that MRP/PLFs have angiogenic activity and also may be growth factors involved in the decidualization of the uterus during mid-gestation. The presence of MRP/PLFs binding in the fetal heart, ribs and liver is still unclear, but it could involve angiogenesis, or growth of certain embryonic tissues (15, 55). MRP/PLFs expression in the hair follicle and wound healing could also suggest similar roles in keratinocyte migration and cell proliferation for MRP/PLFs in the epidermis of the skin (57).

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CHAPTER 2.

EXPRESSION AND PURIFICATION OF RECOMBINANT MRP/PLFS USING 293 HUMAN FETAL KIDNEY CELLS

ABSTRACT:

MRP/PLFs are glycoproteins, belonging to the prolactin-growth hormone family, with an average molecular weight of 34 kDa for PLF1, PLF2, MRP3 and 27 kDa for MRP4. They were originally found to be secreted by trophoblastic giant cells of the mouse placenta during mid-gestation. MRP/PLFs are thought to stimulate angiogenesis in the placenta and the uterus and stimulate uterine growth during mid-gestation. Also, MRP3 is expressed during wound healing and normal hair follicle development. To further study the role of these glycoproteins, a purification method was developed. The 5' end of all the known mature \textit{mrp/plf} cDNAs were PCR-modified to add three features to facilitate expression and purification: the human placental alkaline phosphatase signal sequence, a histidine tag, and an enterokinase recognition site. The modified cDNAs were stably transfected in 293 human fetal kidney cells and the histidine tagged MRP/PLFs accumulated in the conditioned medium. His-MRP/PLFs were separated from contaminating proteins using nickel-chelate then pea-lectin affinity chromatography. His-PLF1 and His-MRP3 were successfully expressed and purified, whereas His-MRP4 did not accumulate in the conditioned medium of stably transfected 293 cells or transiently transfected COS cells. Using enterokinase, the histidine tag was successfully removed from His-MRP3 to release the mature glycosylated form of the MRP3 protein.
INTRODUCTION:

The number of genes in the MRP/PLF family has been estimated at six (1), and four have been cloned and characterized: PLF1 (2), PLF2 (3), MRP3 (4), and MRP4 (5). The protein primary structure deduced from cDNAs suggested that MRP/PLFs are N-glycosylated proteins and are members of the expanding prolactin-growth hormone family (2, 6). Unglycosylated MRP/PLFs have a molecular weight of 22 kDa (7). PLF1, PLF2, MRP3 are secreted glycoproteins with an average apparent molecular weight of 34 kDa. MRP4 is also a secreted glycoprotein but it lacks two of the N-glycosylation consensus sequences of the known MRP/PLFs. MRP4 has an average molecular weight of 27 kDa which is consistent with its lesser degree of glycosylation compared to other MRP/PLF family members (5).

PLF1 was shown to stimulate angiogenesis through the insulin-like growth factor II/mannose-6-phosphate receptor (IGFII/M6P receptor). This binding relies upon the presence of mannose-6-phosphate in the carbohydrate moieties of these glycoproteins (8). During midgestation in the mouse, a MRP/PLF binding activity different from the IGFII/M6P receptor was characterized in the uterus and suggested a role in uterine cell proliferation (9). More recently, the role of MRP/PLFs was broadened by the discovery of MRP3 expression during wound healing and normal hair follicle development (10).

To further study the roles of these proteins, each of the different glycosylated MRP/PLFs needed to be purified in milligram quantities. Initially, Swiss 3T3 cells were stimulated with fibroblast growth factor (FGF), and the clarified conditioned medium was subjected to gel filtration (Biogel P-200) followed by pea-lectin affinity chromatography (11). However, the yield was low, and even though the main MRP/PLF mRNA expressed by FGF-
stimulated 3T3 cells was mainly \textit{plf1}, \textit{mrp3} mRNA was also detected by RT-PCR followed by specific restriction digestion to differentiate between the different MRP/PLF cDNAs (5, 10).

To obtain purified PLF1 and MRP3, a baculovirus expression system using insect cells was developed. However, in insect cells, PLF1 and MRP3 were glycosylated differently compared to placental MRP/PLFs. Such differences could potentially lead to differences in binding to the uterine receptor. Secondly, the eluate from the final purification step contained a contaminant that co-eluted with the recombinant PLF1 and MRP3 as shown by comparing silver stained polyacrylamide gels and Western blots. For both reasons, the baculovirus system for MRP/PLF expression was no longer used (Shogren, Bendickson, and Nilsen-Hamilton, unpublished results).

The purification strategy described here, uses the 293 human fetal kidney cell line (293 cells) as a host for the secretion of recombinant MRP/PLFs. These cells were chosen for several reasons. First, they can be easily transfected; second, they can be adapted to cell suspension; and third, compared to insect cells, these mammalian cells have the ability to glycosylate proteins with a pattern consistent with the endogenous proteins.

All known MPR/PLF recombinant cDNAs were successfully engineered to have a secretion signal sequence, a histidine tag and an enterokinase cleavage site. Stable clones of 293 cells expressing PLF1, PLF2 and MRP3 were generated and a purification scheme was established to purify His-PLF1 and His-MRP3 using nickel chelation and pea-lectin affinity chromatography.
MATERIALS AND METHODS:

Plasmid construction

Plasmids used to transfect 293 cells, with the modified cDNAs for PLF1, PLF2, MRP3 and MRP4 (Figure 1), were obtained by PCR amplification of different plasmids containing

![Figure 1: Structure of the nucleic acid cassette used for stable transfections of 293 cells.](image)

CMV represents the cytomegalovirus promoter. Signal sequence represents the 66 bp of the human placental alkaline phosphatase signal sequence. His represents the sequence for the histidine tag coding for 6 histidines (CAT). EK represents the recognition sequence for the enterokinase coding for the amino acid DDDDK. MRP/PLF represents the cDNA coding for the mature secreted form of PLF1, PLF2, MRP3, or MRP4.

the cDNAs of MRP/PLFs (PLF1: pRSVPLF1, PLF2: pcDNAPLF2, MRP3: MRP3/III/5DS39, MRP4: MRP4/pcDNA3). The 5' end primer Rsal-EK-PLFl contained a Rsal restriction site and the coding sequence for the enterokinase recognition sequence followed by the first 21 nucleotides coding for the first 7 amino acids of the secreted form of PLF1, PLF2 and MRP3. The 5' end primer Rsal-EK-MRP4 was similar to Rsal-EK-PLF1 except it annealed to the cDNA of the secreted form of MRP4. The 3' end primer ZMrpHindIII placed HindIII site into the 3' untranslated region of each MRP/PLFs (Table I).

Table I: Sequences of primers used for plasmid construction

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rsal-EK-PLF1</td>
<td>5'GGGGTACCATGACGATGACGATAAGTTCCCATQTQTQCAATGAGQ 3'</td>
</tr>
<tr>
<td>Rsal-EK-MRP4</td>
<td>5'GGGGTACCATGACGATGACGATAAGTTGCCCATQTGTGCAATQAGG 3'</td>
</tr>
<tr>
<td>ZMrpHindIII</td>
<td>5'GAAAAGCTTQTCAACAACAAATTCAAAAAAG 3'</td>
</tr>
</tbody>
</table>

*Italics represent restriction sites. Underlined is the enterokinase recognition sequence. In bold is the sequence from the MRP/PLFs' genes.*
The PCR reactions were performed using the Expand High Fidelity PCR system (Roche) in a Perkin-Elmer GeneAmp 2400 thermocycler. The amplification program was a denaturation step at 94 °C for 3 min, followed by 30 cycles of amplification (94 °C for 30 s, 60 °C for 30 s, 72 °C for 50 s), and a final elongation step at 72 °C for 5 min.

The expected 686 bp products were gel-purified using the QIAEXII gel extraction kit (Qiagen) and digested with Rsal and HindIII. Restriction digests with XbaI and Rsal of plasmid pcDNAPLF1(His,EK2)PL17 gave a 187 bp fragment containing the human placental alkaline phosphatase signal sequence followed by a histidine tag. The plasmid pcDNA3.1(-) (Invitrogen Life Technologies) was also digested with XbaI and HindIII. After the restriction digests, the digested plasmid pcDNA3.1(-), the 686 bp and the 187 bp fragments were gel purified, ligated together and electroporated in the XL1-blue bacterial strain (Stratagene). The plasmids were isolated and sequenced (DNA Synthesis and Sequencing Facility, ISU). The plasmids containing the modified sequences of PLF1, PLF2, MRP3, and MRP4 were called pcDNA(His,EK)PLF1, pcDNA(His,EK)PLF2, pcDNA(His,EK)MRP3 and pcDNA(His,EK)MRP4, respectively.

Cell culture and transfections

COS cells were grown in DMEM high glucose (Gibco Invitrogen Corporation) supplemented with 10 % calf serum (Summit) and 10 U/ml Penicillin/Streptomycin (Gibco Invitrogen Corporation), at 37 °C in a 10 % CO₂ humidified atmosphere.

COS cells grown in 3 cm tissue culture dishes (Sarstedt) to 50 % confluency were transiently transfected using the DEAE-Dextran method (5). Secreted proteins accumulated in
the medium for 5 to 8 days and the presence of MPR/PLFs was assessed by Western blot.

293 cells were grown in DMEM high glucose supplemented with 5 % calf serum and 10 U/ml Penicillin/Streptomycin. Tissue culture dishes used for 293 cells were treated for 30 min at room temperature before plating with 0.1% Type A gelatin (porcine skin, Sigma) to help the attachment and even spreading of the cells. The cells were grown at 37 °C in a 10 % CO₂ humidified atmosphere. Stable transfections were obtained in 293 cells plated and grown to 50 % confluency in 3 cm tissue culture dishes. Following the manufacturer’s instructions, 0.1 μg of each plasmid were transfected using 0.3 % v/v Lipofectamine™ (Gibco Invitrogen Corporation) in DMEM high glucose for 6 h. The cells recovered overnight and the transfection medium was replaced with fresh growth medium. After 48 h the cells were transferred, using 0.025% trypsin, to a 10 cm tissue culture dish for selection in growth medium containing 300 μg/ml G418 (Sigma). After selection for 14 days, the cells were detached using 0.025 % trypsin and diluted to 15 cells/ml followed by the plating of 0.1 ml in individual wells of a 96 well plate. The cells were grown 3 to 4 weeks and the conditioned medium was collected when it started to acidify (indicated by the phenol red). The cells (estimated to be 20 to 90 % confluent) were detached using trypsin and cryopreserved at - 80 °C. The conditioned medium was analyzed for the presence of the different MRP/PLFs by Western blot.

Point mutation in MRP4 cDNA

Sequencing of pcDNA(His,EK)MRP4 showed that the cloned MRP4 cDNA contained a point mutation at position 155, from the translational start of MRP4 when compared to the
native placental MRP4 cDNA. To obtain the native form of MRP4 in pcDNA3.1(-), this cloned cDNA had to be point-mutated to replace the point mutated adenine at position 155 with a guanine correction. The QuickChange™ method (Stratagene) was used with the following primers. Primer 1: GACACATTTGAATTAGCCGGCAGTTTGTC, Primer 2: GAGACAAACTGCGCGCTAATTCAAATGTGTTC. Plasmids containing the mutation were generated with the following PCR program 1 min at 95 °C, 12 cycles (95 °C 1 min, 55 °C 1 min, 68 °C 13 min), 2 min at 37 °C using the Perkin-Elmer GeneAmp 2400 thermocycler. The mutated cassette containing the signal sequence, the histidine tag and the successfully mutated cDNA was cut out using XbaI and HindIII and recloned into the pcDNA3.1(-) vector and confirmed by sequence analysis (DNA Synthesis and Sequencing Facility, ISU).

**SDS polyacrylamide gel electrophoresis**

Samples to be separated by SDS PAGE were loaded on a 12.5% acrylamide gel and electrophoresis was completed at 35 mA for 3 h in 0.4 M Glycine, 50 mM Tris, 1% SDS pH 8.35. The molecular weight markers were bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), myoglobin (18 kDa), cytochrome c (12.5 kDa).

**Western blot analysis and densitometry**

MRP/PLF containing samples were first resolved by SDS-PAGE. The proteins were transferred on nitrocellulose membrane (Nitrobind 0.45 μm, Osmonics) using a Hoefer TE50 apparatus at 30 V for 12 h at 4 °C. The membrane was stained with 0.2% Ponceau S in 3% trichloroacetic acid to ascertain uniform transfer of proteins to the membrane. After
destaining with water, the membrane was incubated for 30 min at 37 °C in blocking solution (5 % wt/vol nonfat milk in 0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris, 0.02 % wt/vol sodium azide, pH 7.5). The blocking solution was removed and replaced with fresh blocking solution containing rabbit antiserum 89Rb13b raised against MRP/PLF purified from 3T3 cells at a final dilution of 1/200. This antiserum is immunoreactive to PLF1, PLF2, MRP3 and MRP4. After incubation at room temperature for 90 min, the membrane was washed three times (10 min each) in wash buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.5). The first wash contained 1 % Tween 20. The membrane was then incubated for 30 min at room temperature with 8 ng/ml of goat IgG anti-rabbit conjugated to peroxidase (Pierce) in wash buffer. The membrane was washed three times (10 min each) in wash buffer, with the first wash containing 1% Tween 20. The presence of peroxidase activity was detected by enhanced-chemiluminescence using ECL™, (Amersham Pharmacia) and exposure of X-ray film (Kodak).

The intensity of the exposed bands on the X-ray film was determined using a scanning densitometer (Biomed Instruments) measuring directly off the exposed X-ray films.

Silver stain

The different affinity chromatography fractions were resolved by SDS-PAGE, stained in 0.5 % Coomassie blue R-250, 0.25 % isopropanol, 0.1 % glacial acetic acid and destained with 0.1 % isopropanol, 0.1 % glacial acetic acid. Unless indicated otherwise, the following steps were performed at room temperature for 30 min. For enhanced protein staining by silver staining (12), the gel was prefixed in prefix A (50 % methanol, 10 % acetic acid) then in
prefix B (5 % methanol, 7 % acetic acid) followed by fixing in 10 % glutaraldehyde. After thorough rinsing with deionized water for at least one hour with 5 to 6 water changes, the gel was reduced with 5 \( \mu \)g/ml dithiothreitol in water, followed by impregnation with 0.1 % silver nitrate. The silver stain was developed using 0.02 % formaldehyde in 3 % sodium carbonate. Development was stopped after 10 to 15 min by adding directly to the developer 2.3 M citric acid to a 0.1 M final concentration with a pH of 7.

Production of conditioned medium containing His-MRP/PLFs

Roller bottles used for 293 cells were treated, before plating, for 1 h at room temperature with approximately 1 rotation per minute at 37 °C with 0.1% Type A gelatin (porcine skin, Sigma) to help the attachment and even spreading of the cells. The stably transformed 293 cells expressing the different recombinant MRP/PLFs from two confluent 15 cm tissue culture dishes were resuspended in 400 ml of growth medium and seeded in one gelatin treated 1700 cm\(^2\) pleated roller bottle (Corning Science Products), CO\(_2\) was added to the bottle to adjust the pH to approximately 7.3 before the bottle was sealed. The roller bottle was rotated at approximately 1 rotation per minute at 37 °C. When the cells were confluent (after 2 to 4 days), the medium was removed and the cells were washed with 100 ml warm TBS (140 mM NaCl, 8 mM Na\(_2\)HPO\(_4\), 2.7 mM KCl, 1.5 mM KH\(_2\)PO\(_4\), 0.68 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), pH 7.5 ) by rotating the bottle briefly. After removing the TBS, 400 ml of serum free medium was added to the cells, CO\(_2\) was again added, the bottle was sealed and the cells were incubated and rotated as above. After 7 to 8 days the medium was collected and
cell debris was removed by centrifugation at 10,000 x g for 30 min at 4 °C. This provided clarified conditioned medium containing recombinant histidine tagged MRP/PLFs.

**His-MRP/PLFs purification protocol**

The clarified conditioned medium from stably transfected 293 cells was concentrated 10 fold by ultrafiltration through a YM10 membrane (Millipore) using N₂ at 30 PSI at room temperature. A buffer exchange step was done by adding buffer A (50 mM sodium phosphate, 300 mM NaCl, pH 8.0) to reconstitute the original volume. Ultrafiltration was resumed until the conditioned medium was concentrated to 1/20 of its original volume.

The concentrated conditioned medium was further clarified by centrifugation for 30 min at 10,000 x g at 4 °C. Nickel chelate resin (Ni-NTA slurry, Qiagen) was equilibrated in wash buffer A and 1 ml of resin was added to every 20 ml of clarified concentrate. The mixture was rocked overnight at 4 °C to allow binding to the resin. The resin was supported in a poly-prep chromatography column (Biorad), the concentrate was passed through the packed resin and the unbound fraction was collected. The resin was washed with buffer A, followed by buffer B (50 mM sodium phosphate, 300 mM NaCl, pH 6.0). The volume of the these washes was equal to the volume of the unbound fraction. The bound proteins were eluted by sequentially adding 2 ml of a 50 mM sodium phosphate, 300 mM NaCl pH step gradient at pH 6.0, pH 5.5, pH 5.0, pH 4.5, and finally pH 4.0. Bound His-MRP/PLFs was eluted upon addition of pH 4.5 buffer.

The fractions containing His-MRP/PLFs, detected by Western blot, were pooled and loaded on a pea-lectin column equilibrated in buffer C (10 mM Tris, 0.5 M NaCl, pH 8.0).
The column was washed with 10 column volumes of buffer C. Before stopping the flow, 1 column volume of buffer C containing 0.2 M of methyl-α-D-mannopyranoside was added to the column and left at 4 °C overnight. The flow was resumed and 2 ml fractions were collected.

From the pea-lectin column the fractions containing His-MRP/PLFs (detected by Western blot) were pooled and mixed with 5 ml of fresh Ni-NTA resin equilibrated in buffer A and rocked overnight at 4 °C to allow binding. The resin was supported in a poly-prep chromatography column (Biorad), the concentrate was passed through the packed resin and the unbound fraction was collected. The resin was washed with buffer A, followed by buffer B. The volume of the washes was equal to the volume of the unbound fraction. The bound proteins were eluted by sequentially adding 2 ml of a 50 mM sodium phosphate, 300 mM NaCl pH step gradient at pH 6.0, pH 5.5, pH 5.0, pH 4.5, and finally pH 4.0. Bound His-MRP/PLFs was eluted upon addition of pH 4.5 buffer.

At each step, recombinant MRP/PLFs were detected by Western blot and the relative amounts in each fraction were determined by densitometry. Protein contaminants were detected by silver staining. The amount of total protein in the fractions was estimated by using the Coomassie Plus protein assay reagent (Pierce).

**Enterokinase cleavage of the purified proteins**

The fractions containing purified His-MRP/PLFs were pooled, dialyzed against 50 mM ammonium bicarbonate, pH 8.0 at 4 °C and lyophilized. The histidine tagged MRP/PLFs were resuspended in 10 mM Tris-HCl pH 7.5. The histidine tag was cut and removed using 1 unit
of recombinant enterokinase (Novagen) for 20 μg of His-MRP/PLF according to the manufacturer's recommendation. Typical incubations were carried out for 48 to 72 h. To separate the histidine-tag free from the histidine-tagged proteins, Ni-NTA resin was added to the reaction mixture and rocked at room temperature for 1 h. The cut recombinant MRP/PLFs were separated from the nickel column using a spin basket provided with the kit.

RESULTS:

Generation of stable clones expressing recombinant proteins

Protein sequencing of recombinant PLF1 purified from the medium of transfected CHO cells showed that phenylalanine 30 was the first amino acid of secreted PLF1 (13). To facilitate the purification of mature MRP/PLF proteins from transfected 293 cells the cDNAs of these proteins were modified at the 5' end to encode the mrp/pifs cDNA for the mature proteins. In addition the 5' end was sequentially modified to add the strong signal sequence of the human placental alkaline phosphatase (hpAP), a six-histidine tag and an enterokinase recognition site by PCR. The enterokinase sensitive site was added to allow cleavage of the histidine tag to release only the mature secreted form of MRP/PLFs. The cytomegalovirus promoter was used to increase transcription of the expression cassette (Figure 1).

Stably transfected 293 cells containing the vectors for each MRP/PLF were selected by detecting MRP/PLFs in conditioned medium by Western blots. Table II shows that after transfecting the cells with pcDNA(His,EK)PLF1 and pcDNA(His,EK)MRP3, 24% of the G418 resistant clones expressed detectable amounts of recombinant His-PLF1 and His-MRP3, respectively.
TABLE II: Stable transfection percentages for the different His-MRP/PLFs

<table>
<thead>
<tr>
<th>His-MRP/PLFs</th>
<th>Number of wells plated</th>
<th>Number of clones resistant to G418</th>
<th>Numbers of clones expressing His-MRP/PLFs</th>
<th>% clones expressing His-MRP/PLFs</th>
<th>% clones resistant to G418</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-PLF1</td>
<td>288</td>
<td>137</td>
<td>33</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>His-PLF2</td>
<td>192</td>
<td>55</td>
<td>2</td>
<td>4</td>
<td>29</td>
</tr>
<tr>
<td>His-MRP3</td>
<td>288</td>
<td>238</td>
<td>58</td>
<td>24</td>
<td>83</td>
</tr>
<tr>
<td>His-MRP4</td>
<td>288</td>
<td>108</td>
<td>0</td>
<td>0</td>
<td>38</td>
</tr>
</tbody>
</table>

* The pooled clones were cryopreserved before plating in 96 well plates.

The pool of G418 resistant cells transfected with pcDNA(His,EK)PLF2 was cryopreserved prior to diluting and plating in the 96 well plates, which probably resulted in decreased cell viability leading to a decrease in His-PLF2 expressing clones. Clones of 293 cells resistant to G418 were obtained when transfected with the plasmid pcDNA(His,EK)MRP4 suggesting that stably transfected cells were obtained. However, no clones expressed detectable amounts of secreted His-MRP4 by Western blot. This observation led to a further investigation of the putative MRP4 clones.

MRP4 is not detected when expressed with the His-EK constructs

During the construction of the pcDNA(His,EK)MRP4 plasmid, DNA sequencing revealed that the MRP4 cDNA contained a point mutation (position 155 from the start codon) that changed the original glycine codon (GGC) to an aspartic acid codon (GAC). Utilizing the QuickChange method (Stratagene), the original glycine codon was restored. This corrected cDNA was recloned into pcDNA3.1 (-) which was then used for the transfection of 293 cells as described above to obtain stable His-MRP4 expressing clones. Transient transfections in COS cells were used to further investigate the lack of His-MRP4.
accumulation in the conditioned medium of 293 cells stably transfected with pcDNA(His,EK)MRP4.

The plasmid cmv-mini-sis-MRP4, was transiently transfected in COS cells as a positive control for MRP4 expression and detection, as demonstrated by Fassett et al. (5). The rabbit anti-MRP/PLF serum 89Rb13b used for Western blots was shown to detect MRP4 in the conditioned medium of transiently transfected COS cells and of placental extracts (5). COS cells transiently transfected with pcDNA(His,EK)PLF1 could secrete and accumulate His-PLF1 in the conditioned medium. However, similar to what was observed with stably transfected 293 cells, His-MRP4 could not be detected by Western blot in the conditioned medium of COS cells transiently transfected with pcDNA(His,EK)MRP4 (Figure 2).

DNA sequencing showed that Cmv-mini-sis-MRP4 contained the mutated version of MRP4. To rule out the possibility that this point mutation was responsible for the inability to detect recombinant protein in conditioned medium, the mutated version of pcDNA(His,EK)MRP4 was also transiently transfected in COS cells. No accumulation of His-MRP4 in the conditioned medium could be detected by Western blot (data not shown).

Finally, to rule out the possibility of detrimental mutations in the pcDNA vector itself, the expression cassette for (His,EK)MRP4 was reinserted in the pcDNA3.1(–) vector portion of the pcDNA(His,EK)PLF1 construct. No detectable amounts of His-MRP4 could be detected by Western blot in the conditioned medium of COS cells (data not shown).
Figure 2: His-MRP4 cannot be detected in the conditioned medium of transiently transfected Cos cells. Western blot of conditioned medium collected 5 days after COS cells were transiently transfected with 5μl of water: (H2O), pcDNA3.1(-): (Vec), pcDNA(His,EK)PLF1: (His-PLF1), pcDNA(His,EK)MRP4: (His-MRP4) or cmv-mini-sis-MRP4: (MRP4). The numbers represent the amount in μg of plasmid used for the DEAE dextran transfection of 50 % confluent COS cells. The molecular weight markers used are defined in Materials and Methods.

Optimization of culture conditions for maximal secretion

The 293 cell clone secreting His-MRP3 was initially grown in 5 % calf serum. To decrease the amount of contaminating serum proteins for protein purification the clones were switched to serum-free conditions and His-MRP3 accumulation in the conditioned medium was tested. After reaching confluency, the 293 cell clones were rinsed with warm TBS and switched to serum free medium. Recombinant His-MRP3, detected by Western blot, accumulated in the conditioned medium and protein contaminants were detected by silver staining. More His-MRP3 was secreted when the medium contained 5% serum compared to
no serum, but a significant amount of His-MRP3 was still secreted in the absence of serum (Figure 3). The Western blot showed no detectable degradation products for His-MRP3 suggesting that it is stable in the conditioned medium. Similar results were observed for His-PLF1 (data not shown).

![Figure 3: Serum dependency of the secretion of His-MRP3 in the conditioned medium of a 293 clone.](image)

After reaching confluency the cells were washed with warm TBS. Medium containing 0 % or 5 % calf serum was added to the cells. For each time point an aliquot was taken, cell debris was removed by centrifugation, and the supernatant was frozen until use. Panel A: SDS-PAGE of the conditioned medium followed by Coomassie Blue and silver staining. Indicated on the left are the molecular weights of the protein standards defined in Materials and Methods. Panel B: Duplicate samples were run in parallel and His-MRP3 was detected by Western blot. No other bands aside from the ones shown here were detected.

Protein secretion can be stimulated in cells grown in serum free medium by adding a mixture of insulin, transferrin and selenium (14, 15). To test if the cells would accumulate more recombinant proteins in the presence of these three medium supplements, they were added in different combinations. Figure 4 shows more recombinant His-MRP3 accumulated in the conditioned medium in the presence of insulin compared with no insulin regardless of the
presence of transferrin and selenium. Thus, insulin alone was enough to increase the overall accumulation of proteins in the conditioned medium as seen by silver staining.

From these results, the conditions for the highest His-MRP3 secretion with the lowest amount of serum contaminants were established as follows: The cells were grown to confluency in a medium containing 5% serum, washed with warm TBS and switched to serum free medium supplemented with 5 μg/ml of insulin and the cells were incubated 8 days to secrete recombinant His-MRP3. Subsequently it was discovered that insulin at such high concentrations inhibited binding of His-MRP3 to the nickel affinity column (data not shown).

Figure 4: Action of insulin, transferrin and selenium on the accumulation of His-MRP3 in the conditioned medium of stably transfected 293 cells. After reaching confluency the cells were washed with warm TBS and medium containing the different supplements was added. Insulin and transferrin were added at 5 μg/ml and selenium at 5 ng/ml. B: water, I: insulin, T: transferrin, S: selenium, IT: insulin and transferrin, IS: insulin and selenium, TS: transferrin and selenium, ITS: insulin, transferrin and selenium. Panel A: Silver stain of the conditioned medium resolved by SDS-PAGE followed by Coomassie Blue and silver staining. Indicated on the left are the molecular weights of the standards defined in Materials and Methods. Panel B: Duplicate samples were run in parallel and His-MRP3 was detected by Western blot. No other bands aside from the ones shown here were detected.
To prevent problems during purification, His-MRP3 was accumulated in the absence of insulin in the medium. Similar results were obtained with the 293 clone expressing His-PLF1.

**Purification of recombinant His-MRP3**

The recombinant proteins were purified from the conditioned medium of a 293 cell clone expressing His-MRP3 by successive affinity chromatography fractionation on a nickel chelate column, a pea-lectin column, and a second nickel column. Detection of His-MRP3 in the different fractions was performed using Western blot with the rabbit antiserum 89Rb13b. Purity was determined by Coomassie staining followed by silver staining. Typical yields were approximately 0.1 to 0.2 mg of protein per liter of conditioned medium (Table III).

**TABLE III: Purification profile of His-MRP3.**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Fraction Volume (ml)</th>
<th>Total Protein (µg/ml)</th>
<th>MRP Concentration (arbitrary)</th>
<th>Total Protein (mg)</th>
<th>Total MRP (mg)</th>
<th>Purity (%)</th>
<th>Yield (%)</th>
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</thead>
<tbody>
<tr>
<td>Conditioned Medium</td>
<td>4000</td>
<td>35</td>
<td>6</td>
<td>139</td>
<td>5.0</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>After concentration/</td>
<td>215</td>
<td>722</td>
<td>96</td>
<td>155</td>
<td>4.3</td>
<td>3</td>
<td>81</td>
</tr>
<tr>
<td>Before Nickel I</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>After Nickel I/</td>
<td>38</td>
<td>179</td>
<td>201</td>
<td>7</td>
<td>1.5</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>Before Lectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After Lectin/</td>
<td>90</td>
<td>10</td>
<td>64</td>
<td>9</td>
<td>1.2</td>
<td>13</td>
<td>23</td>
</tr>
<tr>
<td>Before Nickel II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>After Nickel II/</td>
<td>14</td>
<td>35</td>
<td>167</td>
<td>0.5</td>
<td>0.5</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>Pooled fractions</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

a: The arbitrary units correspond to the intensity (determined by densitometry) of the bands from the Western blot of figure 5. b: The total amount of His-MRP3 in each fraction was determined by assuming that all the proteins in the "pooled fractions" were MRP (see Figure 5). c: Purity: total MRP/Total protein. d: Yield: MRP/Initial amount of MRP

The recombinant His-MRP3 secreted by the 293 cell clone was recognized by the MRP/PLFs antiserum, indicating that the protein purified is immunologically similar to the
endogenous MRP/PLFs. The expected molecular weight of 35 to 41 kDa observed on the Western blot and silver stained gels (Figure 5) corresponds to the molecular weight of the native placental MRP/PLFs plus another 5 kDa corresponding to the histidine tag. The presence of the tag was confirmed by the specific binding of His-MRP3 to a nickel column. Finally, 293 cells stably transfected with empty pcDNA3.1(-) did not secret any proteins that

![Figure 5: Purification of His-MRP3.](image)

Panel A: Lane 1: conditioned medium before ultrafiltration. Lane 2: Concentrated medium before loading on the nickel column. Lane 3: pooled fractions containing His-MRP3 post-first nickel column and pre-lectin column. Lane 4: Pooled fractions containing His-MRP3 post-lectin column and pre-second nickel column. Lane 5: Pooled fractions containing His-MRP3 after the second nickel-chelate column. Panel A: The different fractions of the purification scheme were loaded at equal volume on SDS-PAGE followed by Coomassie staining and silver stain. The numbers on the side indicate the molecular weight in kDa of protein standards defined in Materials and Methods. Panel B: Duplicate samples were run in parallel and His-MRP3 was detected by Western blot. No other bands aside from the one shown here were detected.
were recognized by the MRP/PLF antiserum. This demonstrates that the protein purified was not a contaminant from the conditioned medium of 293 cells which could cross react with the MRP/PLF antiserum (data not shown). These results provide evidence that the isolated protein is in fact the recombinant His-MRP3 glycoprotein. Using the same purification protocol, His-PLF1 was isolated from the conditioned medium of the 293 clone stably transfected with the pcDNA(His,EK)PLF1 plasmid with yields comparable to the ones for His-MRP3.

Enterokinase treatment of His-EK-MRP3 to obtain histidine tag free MRP3

The pcDNA(His,EK)PLF1, pcDNA(His,EK)PLF2, pcDNA(His,EK)MRP3 and pcDNA(His,EK)MRP4 plasmids were constructed to include an enterokinase cleavage site to allow the removal of the histidine tag. Figure 6, Panel A, shows a time course of enterokinase digestion to determine the optimal incubation time. By scanning densitometry of the film from the Western blot it appeared that within the first 24 hours 33% of the starting protein was lost. At 48 hours, 53% was lost and at 72 hours 65% was lost. Panel B of Figure 6 shows that enterokinase digested MRP3 can be successfully separated from the undigested His-MRP3 by the use of a nickel affinity column to retain any undigested His-MRP3. The typical recovery of digested MRP3 was about 15% of the starting amount of His-MRP3 as determined by Coomassie plus protein assay (Pierce). His-MRP3 incubated under identical buffer conditions without the enzyme did not show any detectable degradation (data not shown).
DISCUSSION:

Recombinant MRP3 and PLF1 were expressed and purified by N-terminal histidine tagging by first concentrating the conditioned medium of transfected 293 human fetal kidney cells by ultrafiltration. The histidine tagged MRP/PLFs were then purified and further concentrated using three affinity chromatography steps (Nickel chelation, pea-lectin, and another Nickel chelation). The histidine tag can be removed by enterokinase digestion releasing the untagged MRP/PLFs. The stably transfected 293 cells expressed a glycosylated form of PLF1 and MRP3, with a molecular weight range similar to the one observed for placental MRP/PLFs. This is the first report that glycosylated recombinant MRP3 has been expressed and purified to homogeneity. The yields obtained by this methodology are
comparable to those reported by Lee and Nathans (16) who purified glycosylated recombinant PLF1 from CHO cells stably transfected with a plasmid construct containing the metallothionein I promoter upstream of the PLF1 cDNA. The advantages of the added histidine tag was demonstrated by the fewer steps used in this method as compared to Lee and Nathans (16). In addition, if binding of the histidine tagged proteins to the MRP/PLF receptors can be demonstrated, it could allow for a convenient in situ detection method utilizing either anti-poly histidine antibodies or a nickel-HRP detection system, both commercially available.

The decision to add a N-terminal histidine tag to the MRP/PLF recombinant proteins, was made by studying the tertiary structure of human growth hormone bound to its receptor (17). Growth hormone is a member of the prolactin/growth hormone family and PLF1 is 21% identical and 35% homologous to human growth hormone deduced cDNAs. The crystal structure of growth hormone showed the N-terminus was less structured and pointed away from the bulk of the molecule, whereas the C-terminus was not as available and was associated in the binding to the receptor (17). In addition, a chimera of human alkaline phosphatase attached to the N-terminus of rat placental lactogen-I was constructed and expressed in 293 cells to detect binding to lactogenic receptors via its phosphatase activity (18). Placental lactogen I is a member of the prolactin/growth hormone family and the chimera secreted by 293 cells bound to the prolactin receptor and stimulated Nb2 cell proliferation via the prolactin receptor. Alkaline phosphatase activity could be detected in situ specifically where placental lactogen I binding was demonstrated (18). The results of these studies showed that the addition of alkaline phosphatase to the N-terminus of placental-lactogen I and the expression in 293 cells were compatible with receptor binding. Other members of the
prolactin/growth hormone family: prolactin-like protein A (19), decidual/trophoblast prolactin-related protein (20) were also successfully engineered with the alkaline phosphatase at their N-termini, suggesting that a N-terminus histidine tag might not disrupt MPR/PLF binding to its receptors.

The histidine tag on His-MRP/PLF can be removed using the enterokinase recognition sequence in case the additional N-terminus amino acid residues would prevent the MRP/PLF proteins from binding to their receptors. The recovery of enterokinase digested MRP3 was very low with the loss primarily occurring during the first 24 h of incubation with enterokinase. MRP/PLFs are stable proteins at room temperature and can accumulate in the conditioned medium of cells. The presence of the histidine tag did not seem to have any deleterious effect as His-PLF1 and His-MRP3 were stable under the purification conditions. His-MRP3 incubated without the enterokinase, under the same conditions used for enterokinase digestion, did not undergo the rapid loss observed when the enterokinase is present (data not shown). No other enterokinase recognition sites are present in the mature MRP/PLFs that would account for the rapid loss of protein during the first 24 hours of digestion. An additional observation (Nelson, unpublished results) indicated that MRP/PLFs purified from 3T3 cells was readily lost by adsorption to the surface of tubes. It is possible that the loss of protein observed during the enterokinase cleavage was due to preferential adsorption of the cut MRP3 to the side of the tubes during the first 24 h of incubation.

Although insulin increased secretion of recombinant His-MRP/PLFs, this increase was compromised by an accompanying insulin-mediated inhibition of His-MRP/PLF binding to the nickel column. This inhibition was directly demonstrated by the addition of 5 µg/ml insulin to the binding reaction inhibiting the binding of His-PLF1 to a nickel column (data not
Nickel ions in solution have been found in complexes with insulin as hexamers and trimers (21). This suggests that insulin with a binding affinity for nickel ions would effectively block histidine tagged protein binding to the nickel chelate column.

The lack of accumulation of His-MRP4 in the conditioned medium of either 293 or COS cells was unexpected. Why MRP4 did not accumulate in the conditioned medium of these cells is unclear, especially knowing that MRP4 is 97% identical at the cDNA level and 91% identical at the protein level to the other known MRP/PLFs. From the transient transfections experiments using COS cells, MRP4 can in fact accumulate in *in vitro* culture medium denoting stability of the protein in these conditions. The point mutation changing the original glycine\(^{52}\) to aspartic acid in the secreted protein is not responsible for the absence of His-MRP4 in the conditioned medium as the mutated His-MRP4 does not accumulate either. The positive control for MRP4 secretion by COS cells was the plasmid cmv-mini-sis-MRP4 used by Fassett *et al* (5). This plasmid contains several features absent in the plasmid construct pcDNA(His,EK)MRP4 used here, including a MRP3 signal sequence followed by a hybrid intron (22) linked to the MRP4 cDNA then followed with the MRP3 3' untranslated region. It is unclear if these added features are responsible for the accumulation of MRP4 in conditioned medium or if the histidine tag specifically prevents MRP4 accumulation compared to the other MRP/PLF proteins. The role of the histidine tag could be tested by transient transfections of COS cells with the MRP4 cDNA without the histidine tag in the plasmid pcDNA3.1(-). If the histidine tag does not prevent the accumulation of MRP4, the role of the different elements of the cmv-mini-sis-MRP4 plasmid could be tested to determine which one(s) allows the accumulation of MRP4 in the conditioned medium.

Purification of glycosylated recombinant PLF1 and MRP3 in milligram quantities will
allow further testing of the roles of MRP/PLFs. The interactions of MRP/PLFs with their receptors is of special interest to the Nilsen-Hamilton laboratory and should shed light on their roles during gestation and wound healing. The two amino acid difference between the MRP3 and PLF1 proteins suggests that both could act through the same receptor. Comparing the plf1, mrp3 and mrp4 promoters, the mrp3 promoter is the only one to be specifically up-regulated by basic fibroblast growth factor in Swiss 3T3 cells (23). It will be interesting to know if the activities of MRP/PLFs are regulated only at the promoter level, or if regulation can also occur at the receptor level.

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CHAPTER 3.

STUDY OF THE ROLES OF MRP/PLFS DURING GESTATION AND BINDING OF PURIFIED MRP/PLFS TO THEIR RECEPTORS

ABSTRACT:

MRP/PLFs are glycoproteins expressed at high concentration during mid-gestation (day 10 to 14 after fertilization) in the placenta. A proposed role for MRP/PLFs during gestation is the stimulation of angiogenesis and uterine cell growth. To provide insights into the potential regulation of uterine cell proliferation by MRP/PLFs, the profiles of the wet weight and DNA content of the uterus were characterized and compared to an existing profile of MRP/PLF expression during gestation. Uterine DNA and wet weight increased rapidly between day 9 and 14 supporting the proposed role for MRP/PLFs in uterine cell growth during this period. Additionally, steroid profiles in the uterus and placenta were also compared to their plasma concentrations and MRP/PLF expression during gestation. In both the uterus and the placenta, estradiol-17β concentrations were high (71 pg/mg in the uterus and 197 pg/mg in the placenta) at gestational day 6 and low from day 8 to day 18 except for a small peak at day 14 (around 40 pg/mg in both uterus and placenta). The progesterone concentrations, in both the uterus and the placenta, were low except for a peak at day 10 (427 pg/mg in the placenta, 142 pg/ml in the uterus). Direct examination of MRP/PLF stimulation by estradiol-17β or progesterone in day 8 and 9 placental minced, organ cultures demonstrated no effect on secreted MRP/PLF levels. Different preparations of purified MRP/PLFs were used to further study the uterine MRP/PLF receptor on primary uterine cell
and organ cultures, and in an *in vitro* day 11 uterine membrane binding assay. No evidence of biological or binding activity to the uterine MRP/PLF receptor was detected. MRP/PLF has also been shown to bind embryonic tissues during gestation. To further investigate whether MRP/PLF binding was due to localized synthesis within the embryo or export from the placenta, Northern blot and RT-PCR analyses were performed. No mRNA for *mrp/plf* could be detected in whole embryos (gestational day 11 to day 19) nor in total RNA from embryonic spines (gestational day 13 to day 19). This data supports the model that MRP/PLFs are not produced by the embryo but instead may be imported from the placenta.

**INTRODUCTION:**

The MRP/PLF family of glycoproteins is secreted by the trophoblastic giant cells during mid-gestation (1). The following studies have suggested that MRP/PLF can function as stimulators of angiogenesis and uterine cell proliferation. For instance, in the zinc finger transcription factors, GATA 2 or GATA 3, knockout mice, the decrease of MRP/PLF secretion from the placenta was shown to correlate with a decrease in decidual neo-vascularization (2). Jackson *et al.*, showed that recombinant PLF1 could stimulate endothelial cell migration (3). Nelson *et al.* demonstrated the presence of a MRP/PLF binding activity from uterine membrane vesicles and demonstrated that the addition of MRP/PLF could stimulate DNA synthesis of primary uterine cells (4). These observations suggest that MRP/PLFs could be involved in the proliferation and neo-vascularization of the decidua during mid-gestation.
The ovarian steroids, estrogen and progesterone are known to play crucial roles in the preparation of the uterus for implantation during gestation (5). Estrogen is thought to mediate its action on the uterus indirectly via epidermal growth factor (EGF) (6, 7), transforming growth factor-alpha (TGFα) (8, 9) and other members of the EGF family (10). Progesterone, stimulates proliferation of the stromal cells of the uterus during the peri-implantation period (11, 12). In addition, progesterone is thought to have an anti-inflammatory effect in the uterus (13) and plays a role in regulating the immune system by decreasing natural killer cell activity (14, 15). Interestingly, estradiol-17β was shown to increase the level of mrp/plf mRNA in sub-confluent BN/L liver cells, suggesting a possible regulation of MRP/PLF expression by steroids (16). However, the reported plasma levels of estradiol-17β and progesterone in the plasma (17) do not correlate with the rapid increase in MRP/PLF mRNA, at gestational day 10 to 14, in the placenta (18). Yet, the concentration of these steroids in the uterus and the placenta during gestation is unknown. Therefore, the levels of estrogen and progesterone throughout gestation were measured in the maternal serum, placenta and uterus using radioimmunoassays (RIA). The accumulation of MRP/PLF in the conditioned medium of placental organ cultures was also measured in response to treatment with progesterone and estradiol-17β.

The uterus undergoes decidualization, at mid-gestation, which is characterized by the rapid multiplication and differentiation of the uterine stromal cell population, a massive infiltration of leukocytes (19, 20) and an increase in vascular permeability (21). However, the overall relationship of uterine DNA content and mass during gestation is unknown and was examined. Measurement of the DNA content and wet weight of the uterus showed a rapid
increase, during mid-gestation, correlating with the expression profile of MRP/PLFs which also increased during mid-gestation. This observation supports the hypothesis that MRP/PLFs could play a role in the decidualization of the uterus which takes place during mid-gestation. Particularly, Fang et al. showed that MRP3 is the highest secreted member of the MRP/PLF family by the placenta (22) and therefore MRP3 was preferentially examined here, for its role in gestation.

Nelson et al. showed that MRP/PLF isolated from 3T3 cells increased thymidine incorporation in uterine primary cell culture indicating an upregulation in cell proliferation (4). To examine the signaling mediated by MRP/PLFs, different preparations of purified MRP/PLFs, including His-PLFl and His-MRP3 (Chapter 2), were tested in binding assays for the MRP/PLF uterine receptor in vivo, utilizing uterine primary cell cultures and minced uterine organ cultures. No increase in thymidine incorporation in primary uterine cell cultures was observed after addition of MRP/PLFs. To verify that recombinant His-MRP3 could bind to the MRP/PLF uterine receptor it was tested for binding to day 11 uterine membrane preparations under the conditions reported by Nelson et al. (4). His-MRP3 was also characterized to test its binding activity to the insulin-like growth factor II /mannose-6-phosphate (IGFII/M6P) receptor in the binding assay developed by Lee et al. (23). The receptor binding assay is based on the observation that recombinant PLF1 could bind to the IGFII/M6P receptor purified from day 16 liver membrane preparations (23). Binding of PLF1 could be competitively blocked by mannose 6-phosphate suggesting that binding occurred via the mannose 6-phosphate site of the receptor.
Additional roles for MRP/PLFs in angiogenesis and DNA synthesis in the embryo itself have been hypothesized. Preliminary data has shown the presence of mrp/plf mRNA in the embryo, suggesting that the MRP/PLF protein detected in the embryonic tissues could be synthesized in the embryo directly (24). MRP/PLF binding in the embryo could be detected in the heart, and endothelial cells of blood vessels from the developing vertebrae and ribs between day 10 and day 18 of gestation. However, mRNA synthesis of mrp/plf was undetectable in this study, suggesting that MRP/PLF protein was not locally synthesized in the embryo (25). The presence of MRP/PLF in embryonic tissues was further investigated by studying the type and gestational profile of mrp/plf mRNA in the embryo.

MATERIALS AND METHODS:

Animals and reagents

Female CF1 mice, from a colony derived from animals originally obtained from Charles River Laboratories, Inc., were maintained as a breeding colony for the Nilsen-Hamilton laboratory in one of the Iowa State University animal facilities, with a 12 h light, 12 h dark cycle and food and water ad libitum. All animals were housed and treated according to current NIH guidelines. The research reported here was conducted in accordance with the standards set forth in the NIH Guide for the Care and Use of Laboratory Animals. Prior approval was obtained from the Iowa State University Committee on Animal Care for all procedures performed on the animals used in these studies. Animals were killed by cervical dislocation or CO₂ asphyxiation before removal of tissues for the described studies. The detection of a copulation plug was used to determine day 0 of gestation.
All tissue culture supplies were from Gibco Invitrogen Corporation, unless otherwise specified. Fetal bovine serum was purchased from Summit Biotechnology. Recombinant IGFII (r-IGFII) was purchased from R&D Systems. The IGFII/M6P receptor was a gift from Dr G. Sahagian (Tufts University School of Medicine, Boston, MA).

Quantitation of uterine DNA during gestation

Uteri were dissected from female mice at various stages of gestation; each sample included both uterine horns. The total weight was recorded and the tissue was frozen in liquid nitrogen. Random sections of the uterus were cut, weighed and homogenized in ice cold 0.5 N perchloric acid using a Tissumizer (Tekmar). The homogenate was centrifuged at 12,000 x g for 2 min at 4 °C, resuspended in 0.5 N perchloric acid and hydrolyzed at 90 °C for 30 min. After centrifugation for 2 min at 12,000 x g and 4 °C, the supernatants were diluted 1/2.5, 1/5, 1/10 and 1/20 in 0.5 N perchloric acid and their DNA content was analyzed using the diphenylamine-colorimetric method (26, 27). A standard DNA curve was made for each assay from a stock of 0.2 mg/ml salmon sperm DNA type III (Sigma) in 0.5 N perchloric acid. Different stocks of DNA standards were normalized to each other by measuring their absorbance at 260 nm. Diphenylamine reagent (1.5 g diphenylamine dissolved in 100 ml of glacial acetic acid and 1.5 ml of concentrated sulfuric acid) was prepared fresh and 0.1 ml of 1.6 % of aqueous acetaldehyde was added just prior to use. One ml of the diphenylamine reagent (with acetaldehyde) was added to 0.5 ml of uterine sample supernatant or DNA standard and the color was developed at 30 °C for 15 to 17 hours. The absorbance of the samples was measured at 600 nm in a DU62 spectrophotometer (Beckman).
Estradiol-17β and Progesterone analysis

Mice at known gestational stages were sacrificed by cervical dislocation. Immediately after death, a sample of systemic blood was collected by heart puncture in a syringe containing heparin at a final concentration between 15 and 50 U/ml. Plasma was prepared by centrifugation for 5 min at 12,000 x g and the supernatant was stored at -20 °C. The uterus was removed and samples of both placental and uterine tissues associated with each fetus were collected, rinsed in TBS (140 mM NaCl, 8 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 0.68 mM CaCl₂, 0.5 mM MgCl₂, pH 7.5), weighed and immediately frozen under liquid nitrogen. The tissue homogenization and radioimmunoassays (RIA) for estradiol-17β and progesterone were done by Carole Hertz from the laboratory of Dr S. Ford (Iowa State University) as part of a collaboration. Tissue samples were homogenized in 1 ml deionized water using a tissue homogenizer (TissueTearor), and frozen at -20 °C until assayed for estradiol-17β and progesterone by RIA. Aliquots of all placental homogenates, and aliquots of all uterine homogenates from a pregnant female were pooled to form a single placental tissue pool and a single uterine tissue pool. An aliquot of each tissue pool (placental and uterine) was then extracted and assayed for estradiol-17β (28) and progesterone (29). Estradiol-17β and progesterone in systemic plasma was also quantitated by RIA (29). Tissue or plasma samples from different days of gestation were evaluated together in the same RIA to decrease errors. Values were normalized to mg of wet weight of tissue.
Minced placental tissue culture

Mice at known gestational stages were sacrificed and placentae were collected and minced to approximately 1-mm³ pieces using two scalpel blades. After rinsing with TBS the minced tissues were centrifuged at 500 x g for 5 min at room temperature. The pellet was rinsed and centrifuged once more. The resulting pellet was resuspended in DMEM high glucose without phenol red supplemented with fetal bovine serum treated with 2 % dextran-coated charcoal to eliminate endogenous steroids, 50 U/ml penicillin/streptomycin, 20 mM HEPES. After addition of estradiol-17β or progesterone, the minced tissues were incubated at 37 °C in 5 % CO₂ for 24 h. The conditioned medium was collected and clarified by centrifugation at 12,000 x g for 5 min at room temperature RT. The presence of MRP/PLFs in the conditioned medium was detected by Western blot.

Western blot analysis and densitometry

MRP/PLF containing samples were diluted in 2 x sample buffer (0.125 M Tris, 20 % glycerol, 0.01 % bromophenol blue, 4 % SDS, pH 6.9) and boiled for 10 min. The samples were resolved by SDS-PAGE on a 12.5 % acrylamide gel and electrophoresis was completed at 35 mA for 3 h in 0.4 M glycine, 50 mM Tris-HCl, 1 % SDS pH 8.35. The molecular weight markers were bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (33 kDa), myoglobin (18 kDa) and cytochrome c (12.5 kDa). The proteins were transferred onto a nitrocellulose membrane (Nitrobind 0.45 μm, Osmonics) using a Hoefer TE50 apparatus at 30 V for 12 h at 4 °C in transfer buffer (25 mM Tris, 190 mM glycine, 20 % methanol, pH 8.3). The membrane was stained with 0.2 % Ponceau S in 3 % trichloroacetic
acid to ascertain uniform transfer of proteins to the membrane. After destaining with water, the membrane was incubated for 30 min at 37 °C in blocking solution (5 % wt/vol nonfat milk in 0.14 M NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 25 mM Trizma pH 7.5, 0.02 % wt/vol sodium azide). The blocking solution was removed and replaced with fresh blocking solution containing rabbit antiserum 89Rb13b (raised against MRP/PLFs purified from 3T3 cells) at a final dilution of 1/200. This polyvalent antiserum is immunoreactive to PLF1, PLF2, MRP3 and MRP4. After incubation at room temperature for 90 min, the membrane was washed three times (10 min each) in wash buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.5). The first wash contained 0.1 % Tween 20. The membrane was then incubated for 30 min at room temperature with 8 ng/ml of goat anti-rabbit IgG conjugated to peroxidase (Pierce) in wash buffer. The membrane was washed three times (10 min each) in wash buffer, with the first wash containing 0.1 % Tween 20. The presence of peroxidase activity was detected by enhanced-chemiluminescence using ECL™, (Amersham Pharmacia) and exposure of X-ray film (Kodak).

**RNA isolation, Northern Blotting and RT-PCR**

Total RNA was isolated from 50 to 100 mg of frozen tissue using TRizol® reagent (Gibco, Invitrogen Corporation) and resuspended in RNAse free water. Total RNA, 15 to 30 µg determined by absorption at 260 nm, was dried using a vacuum centrifuge (SpeedVac, Savant) and the pellet was resuspended in 15 µl of RNA loading buffer (48 % formamide, 0.11 M MOPS, 6.4 % formaldehyde, 5.3 % glycerol, 0.25 % bromophenol blue). The samples were boiled 2 min and loaded onto a 1 % agarose gel, containing 1.7 % formaldehyde,
0.5 μg/ml ethidium bromide. Electrophoretic separation was completed at 60 V for 3 h in 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA pH 7.0. The gel was rinsed at room temperature 15 min in deionized water followed by 15 min in 50 mM NaOH. The RNA was transferred to Zeta-Probe® GT Blotting membrane (Bio-Rad) by alkaline capillary transfer overnight in 50 mM NaOH. The membrane was washed 10 min with 2X SSC (1 X SSC: 0.15 M NaCl, 15 mM sodium citrate pH 7.0) and baked 15 min at 80 °C under vacuum. Before probing, the membrane was pre-hybridized at 47 °C for 6 h with 100 μg/ml yeast tRNA and salmon sperm DNA, boiled 2 min and chilled on ice, in 0.6 M NaCl, 40 mM NaH₂PO₄, 50 mM EDTA, 7 % SDS, 1 % PEG 20000, 40 % formamide, 0.1 % NP40, pH 7.4. A ³²P-labeled mrp/plf probe was generated by using the Multiprime DNA labeling system (Amersham Pharmacia), mrp3 cDNA and 3000Ci/mmol α-³²P-dCTP (ICN). Unincorporated dNTPs were separated from the labeled probe by gel filtration using a micro bio-spin P-30 Tris chromatography column (Bio-Rad). The probe in 10 mM Tris pH 7.4 was boiled 5 min and chilled on ice before adding to the blot in the pre-hybridization solution for a 16 h incubation at 47 °C. The membrane was washed twice for 15 min with 0.5X SSC, 0.1 % SDS at room temperature followed by 2 washes for 15 min with 0.2X SSC, 0.1 % SDS at 47 °C. The membrane was dried and exposed to a PhosphorImager screen at room temperature. The screen was scanned and quantitated using a PhosphorImager 400A and the ImageQuant 3.3 software (Molecular Dynamics).

The "house-keeping" gene glyceraldehyde-3-phosphate dehydrogenase (gpdh) was used to normalize RNA loading. Before reprobing, the ³²P-mrp/plf probe was removed from the membrane by boiling the membrane twice for 3 min in 0.1 X SSC, 0.1 % SDS. The
stripped membrane was pre-hybridized, hybridized with a $^{32}$P-labeled gpdh probe, washed and exposed to a PhosphorImager screen as described above.

**RT-PCR using the SuperScript™ One-Step RT-PCR kit from Invitrogen Life Technologies** was performed on 1 µg of total RNA. Mrp/plf cDNAs were amplified using 0.2 µM of the primers Del (5’ TAAGCCTGGGTAGGACTCTGC 3’) and Ue5 (5’ TTCAGAAGCAGAGC ACATGAAA 3’). Gpdh cDNA was amplified using 0.2 µM of the primers GPDH5’ (5’ TGT GGATGGCCCCTCTGGAAA 3’) and GPDH3’ (5’ GTTTCTTACTCCTGGAGGC 3’). The cDNA synthesis was done at 50 °C for 30 min followed by a pre-denaturation step at 94 °C for 2 min. The PCR amplification consisted of 40 cycles (94 °C for 15 s, 60 °C for 30 s, 72 °C for 50 s) and a final extension step at 72 °C for 10 min using the DNA engine Opticon (MJ Research). The RT-PCR products were resolved on a 1.5 % agarose gel, containing 0.5 µg/ml ethidium bromide, using TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA pH 8.5) at 80 V for 1 h.

**Primary mice uterine cell and minced uterine cell cultures**

Primary uterine cells were isolated using two different methods. The first was developed by Rosenzweig (Nilsen-Hamilton laboratory) and is a modified version of an earlier method (30). The uteri were removed from the mice and separated from the placentae and embryos in sterile conditions. The uteri were rinsed in Ca$^{2+}$, Mg$^{2+}$ free Hank’s balanced salt solution (HBSS), cut in smaller pieces and incubated in 0.25 % Trypsin, 1 mM EDTA in TD at 4 °C for 12 h in a 50 ml conical tube (Corning). The trypsin solution was discarded and the tissue was left in the tube and incubated at 37 °C for 1 h. The uteri were covered with HBSS
and vortexed at the highest setting for 5 min. The HBSS was collected and the remaining tissue was rinsed with fresh HBSS. Both fractions were collected and centrifuged at $1,000 \times g$ for 10 min. The cell pellet was vigorously resuspended with HBSS containing 0.3 % bovine serum albumin (Sigma). The clusters of epithelial cell settled at $1 \times g$ for 10 min in a narrow tube at $37^\circ C$. The supernatant fraction, enriched with the stromal cells, was transferred to a fresh tube and centrifuged for 5 min at $1,000 \times g$. The cell pellet was resuspended in growth medium (DMEM high glucose supplemented with 10 % heat-inactivated fetal bovine serum, 20 U/ml penicillin/streptomycin, 10 ng/ml bovine insulin) and the cells were seeded in a 24 well tissue culture plate at a concentration of $2 \times 10^5$ cells per well. Half of the medium was replaced with fresh growth medium every 48 h. After 3 to 4 days of growth at $37^\circ C$ in 10 % CO$_2$, the cells were confluent. Growth factor additions were made by replacement with fresh growth medium.

The second method for isolating uterine stromal cells was done according to McCormack and Glasser (31). The uteri were isolated, cut in smaller pieces and washed as described above, then incubated in a 50 ml conical tube (Corning) with 0.5 % Trypsin, 2.5 % pancreatin in HBSS for 90 min at 4 °C followed by 30 min at room temperature. The tissues were vortexed 15 s at setting 5 (Fisher vortex genie 2), the supernatant containing the epithelial cells was removed and the remaining tissue pellet was rinsed with HBSS. The tissue pellet was transferred to a 15 ml conical tube (Corning) containing five sterile 5-mm glass beads and 0.25 % Trypsin, 1 mM EDTA in TD. The tissues were incubated at $37^\circ C$ for 15 min with 10 s vortexing at setting 5 (at the beginning, middle and end of the incubation). The cells were centrifuged and washed once with HBSS at $1,000 \times g$ for 10 min. The stromal
cell enriched pellet was resuspended in growth medium and plated as described above at 2 x 10^5 cells per well.

The method used for minced uterine cell cultures was developed by Rosenzweig (Nilsen-Hamilton laboratory). Each fetus *in utero* were separated from each other and the uterine section was separated from the fetus and associated placenta. Each section of uterine tissue was placed in an individual well of a 24 well tissue culture plate containing DMEM high-glucose supplemented with 2% fetal bovine serum, 50 U/ml penicillin/streptomycin and 20 mM HEPES pH 7.5. The uterine tissue were maintained in culture medium at 37 °C in 5% CO₂.

**Thymidine incorporation assay**

Primary uterine cell cultures were prepared according to the protocols described above. The cells were plated on coverslips prepared by successive boiling in Calgon (8.8 g/l Calgon, 79 g/l sodium metasilicate), rinsing in distilled water, boiling 10 min in distilled water, rinsing in distilled water, then a final rinse in absolute ethanol. After drying the coverslips were heat baked sterilized for 4 h at 350 °C.

When the primary uterine cell cultures were confluent, the medium was replaced with fresh growth medium containing different growth factors. The primary uterine cells were incubated for 26 h followed by an additional 4 h pulse with 8 μCi/ml of methyl-³H-thymidine (ICN). Upon completion of the incubations, the medium was removed, the cells were washed twice with 0.5 ml of TBS followed by fixation overnight at room temperature with 3.7% formaldehyde in 85 mM NaCl, 0.1 M Na₂SO₄. After removal of the fixative, the cells were
incubated in 0.5 ml of 10 % trichloroacetic acid (TCA) at 4 °C for 30 min. The cells were rinsed with 1 ml of 95 % ethanol followed by a 5 min dehydration step in 1 ml of 95 % ethanol. The cell-covered coverslips were removed and placed in scintillation vials to dry for 1 h. Five ml of Scintiverse BD cocktail (Fisher) was added and the tritium incorporated by the cells on coverslips was detected in a Liquid Scintillation Analyzer (Packard 1900TR).

For thymidine incorporation in minced uterine tissue culture, 8 μCi/ml ³H-thymidine was added with MRP/PLFs directly after the tissue was placed in growth medium. After 30 h the growth medium was removed and the tissues were rinsed twice for 10 min in 0.5 ml TBS and 0.5 ml of 10 % trichloroacetic acid for 30 min at 4 °C. The tissues were rinsed in 1 ml of 95 % ethanol followed by a 30 min dehydration step in 1 ml of 95 % ethanol. After removing the ethanol the tissues were air dried overnight, weighed and placed in a scintillation vial with 5 ml Scintiverse BD cocktail. The thymidine incorporated in the tissues was detected in a Liquid Scintillation Analyzer (Packard 1900TR).

²¹²iodine labeling of MRP3, r-IGFII, IGFII/M6P receptor

Purified His-MRP3 or MRP3 (Chapter 2), r-IGFII and IGFII/M6P were iodinated using Iodo Beads (Pierce). Typically, 25 to 50 μg of purified protein was labeled with 0.25 to 0.5 mCi of ²¹²Iodine (ICN) for 15 min using one bead. Unincorporated iodine was separated from labeled proteins by size exclusion chromatography using a 10-DG column (Bio-Rad) equilibrated with 25 mM Tris pH 7.6. Iodinated proteins were eluted with 25 mM Tris pH 7.4 into 1 ml fractions, for His-MRP3 and MRP3, BSA was added to the fractions at 0.1% final to stabilize the iodinated proteins. The fractions containing the iodinated protein were pooled.
and purity was determined using SDS-PAGE and autoradiography. The iodinated proteins were analyzed by TCA precipitation before use to determine the total precipitable counts.

**Membrane isolation and receptor binding assay**

Membrane preparations of day 11 uterus were prepared according to Nelson *et al.* (4) and membranes from day 16 livers were prepared according to Lee and Nathans (23). Briefly, uterus or liver tissue were dissected and rinsed in 0.3 M sucrose with all subsequent steps performed at 4 °C. The tissues were homogenized using the Tissumizer (Tekmar) at a setting of 50 for 4 min followed by 1 min at full speed. The homogenate was centrifuged at 3,000 x g for 20 min and the supernatant obtained was further centrifuged at 10,500 x g for another 20 min. This supernatant was then centrifuged at 100,000 x g for 90 min. The pellet containing the uterine membranes was resuspended in 250 mM Tris-HCl, 10 mM MgCl\(_2\), pH 7.6. The pellet containing the liver membranes was resuspended in 25 mM Tris-HCl, 10 mM MgCl\(_2\), pH 7.6. The total protein content was determined using the Coomassie Plus Protein assay (Pierce) and both membrane preparations were aliquoted and stored at -80 °C.

The receptor binding assays on uterine membranes were done according to the method described by Nelson *et al.* (4). Briefly, 0.1 to 0.5 mg/ml of uterine membrane was incubated with \(^{125}\text{I}-\text{His-MRP3}\) or \(^{125}\text{I}-\text{MRP3}\) in Tris buffer-250, (250 mM Tris-HCl, 10 mM MgCl\(_2\), pH 7.6) containing 1 % bovine serum albumin, 10 mM mannose 6-phosphate, 2 mM NaCl, 0.08 mM CaCl\(_2\) at RT for 90 min. Specific binding was determined by adding 100-fold excess unlabeled His-MRP3 or MRP3 to the reaction mixture. One ml of cold Tris buffer-250 was added and the diluted mix was filtered under vacuum on Durapore filters (GVWP 02500,
0.22 μm, Millipore). The filters were washed 6 times with cold Tris buffer-250. The filters were dried 1 h at RT, 5 ml of Scintiverse BD cocktail (Fisher) was added and the $^{125}$Iodine counts were measured using a Liquid Scintillation Analyzer (Packard 1900TR).

The liver membrane binding assay was done according to Lee and Nathans (23). Briefly, 2 to 100 μg/ml of liver membrane was incubated with $^{125}$I-His-MRP3, $^{125}$I-MRP3 or $^{125}$I-IGFII in Tris-HCl pH 7.6 containing 5 mg/ml bovine serum albumin, 6 mM EDTA, 0.04 mM MgCl$_2$ at 4 °C for 12 to 18 hours. Specific binding was determined by adding 10 mM mannose 6-phosphate or 10 to 100 fold non-labeled IGFII to the reaction mixture. The binding reaction was filtered under vacuum on Durapore filters (GVWP 02500, 0.22 μm, Millipore) or GF/C glass microfibre filters (Whatman). The filters were washed 5 times with 10 ml of cold Tris-HCL, pH 7.6. The filters were dried 1 h at RT, 5 ml of Scintiverse BD cocktail (Fisher) was added and the $^{125}$Iodine counts were measured using a Liquid Scintillation Analyzer (Packard 1900TR).

Mannose 6-phosphate detection on glycoproteins by overlay assay

Detection of mannose 6-phosphate on glycoproteins was done according to Valenzano et al. (32). The samples were diluted in 2 x SDS-PAGE sample buffer without β-mercaptoethanol and heated at 50 °C for 10 min. The samples were electrophoresed on a 12.5 % acrylamide gel at 35 mA for 3 h in 0.4 M glycine, 50 mM Tris, 1 % SDS, pH 8.35. The molecular weight markers, bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), myoglobin (18 kDa), cytochrome c (12.5 kDa), were treated like the above samples. The separated proteins were transferred onto nitrocellulose membrane
(Nitrobind 0.45 μm, Osmonics) using a Hoefer TE50 apparatus at 30 V for 12 h at 4 °C in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH 8.3). The membrane was air dried and baked in a vacuum oven for 2 h at 80 °C. The membrane was then stained with 0.2% Ponceau S in 3% trichloroacetic acid to ascertain uniform transfer of proteins to the membrane. After destaining with water, the membrane was blocked for 1 h by rocking at 4 °C in blocking buffer (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 0.2% Tween 20, 10 mg/ml BSA, 5 mM Na β-glycerophosphate pH 7.2). The blocking buffer was replaced with fresh blocking buffer containing 0.4 ng/ml ¹²⁵I-IGFII/M6P receptor and rocked at 4 °C for 8 h. For the blots performed in the presence of competitors, the labeled ¹²⁵I-IGFII/M6P receptor was pre-incubated in blocking buffer containing mannose 6-phosphate (10 mM) or r-IGFII (0.75 μg/ml), at 4 °C for 1 hour. Nonspecific binding to the membrane was removed by washing the blots 10 times (30 s each) with 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 0.2% Tween 20, pH 7.2. The membranes were dried, wrapped in Saran Wrap and exposed to a PhosphorImager screen and scanned using a Molecular Dynamics PhosphorImager 400A.

RESULTS:

The wet weight and DNA content of the uterus correlate with the presence of MRP/PLFs

MRP/PLFs are thought to be growth factors for mid-gestational uterine cells because of their ability to stimulate DNA synthesis in primary uterine cell cultures (4). The profiles of the wet weight and DNA content of whole uteri were measured during gestation to better understand the biology of the mouse uterus and to establish a possible correlation with
MRP/PLF expression. Mouse uteri were dissected from pregnant female at different days of gestation and weighed. A two-fold increase in the uterus wet weight from day 0 to day 8 was observed, followed by a 4-fold increase from day 8 to day 14. From day 14 to day 16, the wet weight of the uterus stabilized followed by a further increase of 1.4-fold until parturition (Figure 1A). Figure 1B displays the profile of total DNA per uterus during gestation and demonstrates that the change in total DNA paralleled the profile of the uterus wet weight. Prior to day 8, the amount of DNA per uterus did not increase significantly, but from day 9 to day 14 the amount of DNA increased by nearly 3-fold. After day 14, there was no significant increase in the total DNA in the uterus until birth whereas the wet weight of the uterus still increased. Uterine total DNA content increased between days 9 and 14 by 0.2 μg DNA/uterus/day, which corresponds to an increase of 15 % of the uterine mass per day. An overlay of the expression profile of MRP/PLF proteins during gestation reveals a positive correlation between the rapid increase in both wet weight and DNA amounts and the peak of MRP/PLF secreted by the placenta (Figure 1 A&B).
Figure 1: Wet weight and total DNA in the mouse uterus during gestation.

In panel A and B, the hatched line (---) represents the expression profile of MRP/PLF proteins in the placenta.
Panel A: the number of uteri collected at different days of gestation and weighed was: 1 (day 2), 3 (day 6), 4 (day 4), 5 (days 1, 3, 8, 10, 12, 14), 6 (days 5, 7, 16), 10 (day 17), 11 (days 15), 12 (day 9), 17 (day 13), 26 (day 0), 59 (day 11). Panel B: the amount of DNA per uteri was determined as described in Materials and Methods. The number of uteri for each day was: 2 (days 1, 4, 5, 6, 7, 8, 10), 3 (days 0, 12), 4 (days 11, 14, 16, 18, 19), 5 (day 17), 6 (days 9, 15, 17), 7 (day 13). The error bars represent +/- 1 standard deviation.

Estradiol-17β and progesterone profiles in placenta, uterus and plasma during gestation

In sub-confluent BN/L liver cells, the level of mrp/plf mRNA increased upon addition of estradiol-17β, suggesting a possible regulation of MRP/PLF expression by steroids (16).
However, the reported plasma levels of estradiol-17β in the plasma (17) do not correlate with the rapid increase in MRP/PLF mRNA, at gestational day 10 to 14, in the placenta (18). The collective sum of many studies investigating the factors regulating uterine growth in immature or ovariectomized mature female rats and mice have shown that estrogen and progesterone are major regulatory factors (10, 33-35). MRP/PLF could be regulated by steroids expressed locally in the uterus or the placenta. However, no data on the local concentrations of estradiol-17β and progesterone was available for these tissues during gestation. Therefore, the concentrations of estradiol-17β and progesterone were measured by RIA in the uterus and placenta of CF-1 mice at various stages of gestation.

Figure 2A shows that the levels of estradiol-17β in the uterus and the placenta were less than 1 pg/mg throughout gestation except for 2 distinct peaks, one at day 6 (71.2 ± 27.3 and 197.3 ± 36.7 pg/mg in the uterus and the placenta, respectively) and the other at day 14 of gestation (33.8 ± 9.0 and 45.2 ± 12.9 pg/mg in the uterus and placenta, respectively). The concentration of estradiol-17β in the plasma was highest in day 0 pregnant mice (49.2 ± 2.9 pg/ml) and decreased to 20 pg/ml until day 14 when the plasma level steadily increased to 55.6 pg/ml at day 18.

The levels of progesterone were also measured in the same tissues. Figure 2B shows that the progesterone levels in the placenta and the uterus were approximately 50 pg/mg for both tissues during gestation except for a peak on day 10 of gestation (427.0 ± 94.5, 142.4 ± 27.4 pg/mg in the placenta and uterus respectively). In the plasma, low levels of progesterone on day 0 (4.9 ± 0.2 ng/ml) initially peaked on day 8 (27.0 ± 3.2 ng/ml) and then decreased to day 10 levels (19.3 ± 0.6 ng/ml). The concentration increased to its maximum on
day 12 (42.1 ± 6.3) followed by a decrease to day 18 (27.34 ± 1.7 ng/ml). Both gestational profiles for estradiol-17β and progesterone in the plasma of the CF1 mice were similar to the ones previously reported for Swiss mice (17).

Figure 2: Estradiol 17β and progesterone levels in the uterus, placenta and serum during gestation. The levels of estradiol during gestation (panel A) were measured by RIA in the uterus (○), the placenta (▲) and plasma (■) as described in Materials and Methods. The levels of progesterone were measured by RIA in the same tissues during gestation (panel B). For both panels, the number of samples contributing to each average value was as follows: 2 (days 0, 6, 10, 18), 3 (days 8, 12, 14, 16).
Effect of estradiol-17β and progesterone on MRP/PLFs production by the placenta

The peak of progesterone observed in the placenta and the uterus on day 10 suggested that progesterone could be involved in up-regulating the secretion of MRP/PLFs by the placenta. To examine whether progesterone could in fact stimulate the secretion of MRP/PLFs from the placenta, day 8 and 9 minced placental tissue cultures were treated with $10^{-6}$ to $10^{-8}$ M progesterone. These concentrations were similar to the ones measured for the placenta in Figure 2B. Secreted MRP/PLFs were detected in the placental tissue cultures after 24 h by Western blot of conditioned medium (Figure 3). There was no observed increased in

![Figure 3: Western blot of conditioned medium of placental minces stimulated with steroids.](image)

*Figure 3: Western blot of conditioned medium of placental minces stimulated with steroids.* Western blot showing MRP/PLFs present in the conditioned medium of minced placental cultures (day 9 of gestation; day 8 not shown) treated with 0, $10^{-6}$, $10^{-7}$ or $10^{-8}$ M of progesterone. Each concentration was tested in triplicate (a, b, c) and the same volume of conditioned medium was used to detect MRP/PLFs on Western blot. The molecular weight markers used are defined in Materials and Methods.
MRP/PLF accumulation in the conditioned medium of day 8 or 9 minced placental tissue cultures after progesterone treatment. Additionally, Estradiol-17β did not have any effect on the secretion of MRP/PLFs in the conditioned medium of day 8 and 9 minced placental tissue cultures (data not shown).

\textit{\textsuperscript{3}H-thymidine incorporation of uterine cells stimulated by MRP/PLFs}

Different preparations of purified MRP/PLFs were tested for binding to the MRP/PLF uterine receptor \textit{in vivo} (uterine primary cell cultures and minced uterine organ cultures) to define the conditions for MRP/PLF mediated signaling. These MRP/PLF preparations were tested for their ability to stimulate \textsuperscript{3}H-thymidine incorporation of gestational day 11, uterine primary cell cultures isolated using the two different methods described in Materials and Methods. One MRP/PLF preparation was isolated from conditioned medium of FGF-stimulated Swiss 3T3 cells (36). Another preparation was isolated from the conditioned medium of FGF-stimulated BALB/c normal liver (BN/L) cells (4). Finally, two different preparations were isolated from the conditioned medium of stably transfected 293 cell clones secreting recombinant His-PLFl (Chapter 2). No reproducible increase in thymidine incorporation could be demonstrated in uterine primary cell cultures (isolated with either method) treated with MRP/PLFs isolated from 3T3 or BN/L cells nor with His-PLF1 isolated from transfected 293 cells (data not shown).

MRP/PLFs isolated from BN/L cells were previously shown to stimulate \textsuperscript{3}H-thymidine incorporation in gestational day 11 minced uterine tissue cultures (Rosenzweig, unpublished data). Unlike isolated primary uterine stromal cell cultures, minced uterine tissue cultures
contain multiple cell populations that might respond to MRP/PLF-stimulation and show increased thymidine incorporation. Therefore, MRP/PLF preparations isolated from 3T3, BN/L cells and recombinant His-PLF1 and MRP3 were re-tested on minced uterine tissue cultures. Figure 4 shows the absence of a reproducible increase in thymidine incorporation by gestational day 11 minced uterine tissue cultures treated with different concentrations of recombinant MRP3 (without histidine tag). Similarly, no MRP/PLF preparations from 3T3 or BN/L cells tested stimulated $^3$H-thymidine incorporation in day 11 minced uterine tissue cultures.

![Figure 4: $^3$H-Thymidine incorporation of day 11 minced uterus tissue cultures treated with MRP3.](image)

Minced uterine tissue cultures were treated with different concentrations of recombinant MRP3 (without a histidine tag) for 30 h in the presence of 8 μCi/ml $^3$H-thymidine. The amount of radioactivity in each piece of uterus was normalized to the dry wet of the tissue. DNA synthesis is expressed as fold over control (cpm treatment / cpm control). The error bars represent the standard deviation of four replicates.
In vitro membrane binding assays to the MRP/PLF receptors

To examine if His-MRP3 purified in Chapter 2 could bind to the putative MRP/PLF uterine receptor, His-MRP3 was iodinated and tested for binding to gestational day 11 uterine membrane preparations under the conditions reported by Nelson et al. (4). In that report, 1 mM Mannose 6-phosphate did not compete with the \(^{125}\text{I}\)-MRP/PLF for binding to these membrane preparations indicating that the binding activity was not due to the IGFII/M6P characterized for maternal liver (4). For the following experiments, several concentrations of His-MRP3 or MRP3 (Chapter 2), and gestational day 11 uterine membranes were used to fall within the range of concentrations previously reported to show binding activity. Binding of \(^{125}\text{I}\)-His-MRP3 to gestational day 11 uterine membranes was detected in the presence of 10 mM mannose 6-phosphate but competition with non-labeled His-MRP3 did not decrease the number of counts suggesting non-specific binding to the uterine membranes or to the Durapore filters used in that assay. Similarly, only non-specific binding was observed when \(^{125}\text{I}\)-MRP3 (without the histidine tag) was tested on gestational day 11 uterine membrane preparations (data not shown).

Binding to the IGFII/M6P receptor was used to determine the glycosylation state of His-MRP3 and MRP3. There is only a two amino acid difference between PLF1 and MRP3 which does not affect the number of putative N-glycosylation sites present on MRP3. The amount of glycosylation of His-MRP3, determined by the estimation of the molecular weight determined by SDS-PAGE, was similar to wild type MRP/PLF (Chapter 2). Binding of His-MRP3 to the IGFII/M6P receptor present in day 16 pregnant maternal liver membrane preparation was performed according to Lee and Nathans (23). First, the integrity of the
gestational day 16 maternal liver membrane preparation was verified using labeled insulin

growth factor II (IGFII) which also binds to the IGFII/M6P receptor via a different binding site
(37). Figure 5 shows that under the experimental conditions reported by Lee and Nathans (23)

\[ ^{125}\text{I-IGF-II} \]

can bind to gestational day 16 maternal liver membranes. The addition of 100 fold
cold IGF-II partially competed the binding of the labeled IGF-II to the membranes. Moreover,
although the GF/C filters gave higher background counts, compared to the Durapore filters,
the GF/C filters also showed a greater difference between binding of \[ ^{125}\text{I-IGF-II} \] plus or minus
cold IGF-II.

His-MRP3 (Figure 5) and MRP3 (data not shown) non-specific binding to gestational
day 16 maternal liver membrane preparations was detected using GF/C or Durapore filters.
Several concentrations of His-MRP3 or MRP3 and gestational day 16 liver membranes were
used and were within the range of concentrations reported by Lee and Nathans (23).

**Overlay assay to detect mannose 6-phosphate on His-MRP3**

The absence of binding of His-MRP3 to the day 16 liver membranes suggested the
possibility that recombinant His-MRP3 isolated from 293 cells did not have mannose 6-
phosphate. The overlay assay developed by Valenzano et al. (32) showed that the bovine
IGFII/M6P receptor can bind to His-MRP3 (Figure 6 A). Receptor binding by His-MRP3 was
competed off by the presence of mannose 6-phosphate in the overlay solution (Figure 6 B).
Duplicate samples for the overlay assay were used on a Western Blot to ascertain the presence
of the His-MRP3 (Figure 6 C). Unexpectedly, two bands were present, one at approximately
Gestational day 16 liver membrane receptor binding assay was outlined in Materials and Methods. In a final volume of 0.5 ml, 12 μg of the membrane preparations and ligand $^{125}$I-IGF-II (IGFII) and $^{125}$I-His-MRP3 (MRP3) were incubated with or without competitor, IGF-II (IGFII), Mannose 6-phosphate (M6P), His-MRP3 (MRP3) at 4 °C overnight. The mixture was filtered under vacuum using Whatman GF/C filters (glass fiber) or Millipore Durapore GVWP filters (PVDF) and washed 5 times with 10 ml of 25 mM Tris, HCl, pH 7.6. The cpm retained by the filters are represented as total cpm retained (grey bars) or as % of retained cpm over total cpm (striped bars). The filters used were G: Whatman GF/C, D: Millipore Durapore GVWP). Each condition was repeated in triplicate, and the error bars represent +/- 1 standard deviation.

Figure 5: Receptor binding assay on gestational day 16 maternal liver membrane vesicles

Gestational day 16 liver membrane receptor binding assay was outlined in Materials and Methods. In a final volume of 0.5 ml, 12 μg of the membrane preparations and ligand $^{125}$I-IGF-II (IGFII) and $^{125}$I-His-MRP3 (MRP3) were incubated with or without competitor, IGF-II (IGFII), Mannose 6-phosphate (M6P), His-MRP3 (MRP3) at 4 °C overnight. The mixture was filtered under vacuum using Whatman GF/C filters (glass fiber) or Millipore Durapore GVWP filters (PVDF) and washed 5 times with 10 ml of 25 mM Tris, HCl, pH 7.6. The cpm retained by the filters are represented as total cpm retained (grey bars) or as % of retained cpm over total cpm (striped bars). The filters used were G: Whatman GF/C, D: Millipore Durapore GVWP). Each condition was repeated in triplicate, and the error bars represent +/- 1 standard deviation.

35 kDa and the other one approximately 50 kDa. The higher molecular weight band appears to be very poorly detected by the IGFII/M6P receptor. Figure 6 D showed that the presence of the high molecular weight band for His-MRP3 in the Western blot is due to the absence of β-mercaptoethanol. These results suggest that at least 50 % of the His-MRP3 isolated contained detectable amounts of mannose 6-phosphate. Recombinant-IGF-II was also used as a control, and binding of the receptor was detected and competed off by the presence of r-IGF-II (data not shown).
Figure 6: Overlay assay showing the presence of mannose 6-phosphate on His-MRP3

The different fractions from the purification scheme used in Chapter 2 were tested for the presence of mannose 6-phosphate using the overlay assay as described in Materials and Methods. In panels A, B and C Lane 1: Conditioned medium before ultrafiltration, Lane 2: Concentrated medium before loading on the primary nickel column, Lane 3: Pooled fractions containing His-MRP3 post-first nickel column and pre-lectin column, Lane 4: Pooled fractions containing His-MRP3 post-lectin column and pre-secondary nickel column. Lane 5: Pooled fractions containing His-MRP3 after the second nickel column. Duplicates of the different fractions were loaded at equal volumes on 12.5 % SDS-PAGE. Panel A shows the overlay assay using $^{125}$I-IGFII/M6P receptor, panel B shows the overlay assay using $^{125}$I-IGFII/M6P receptor pre-incubated with 10 mM mannose 6-phosphate. Panel C shows the Western blot using antiserum 89Rb13b raised against MRP/PLFs. Panel D shows a Western blot of the pooled fractions containing His-MRP3 after the second nickel column resuspended in 2 x loading buffer with or without β-mercaptoethanol (+BME, -BME respectively) before heating the samples at 50 °C and loading them on 12.5 % acrylamide gel. The numbers on the side indicates the molecular weight in kDa of protein standards defined in Materials and Methods.
Detection of MRP/PLF mRNA in embryonic spine

Dudley reported the detection of *mrp/plf* mRNA in gestational day 11 embryonic tail and in gestational day 17 embryonic spines by Northern blot and RT-PCR (24). However, Jackson *et al.* showed that although recombinant PLF1 could bind to the embryonic spine using $^{125}$I-labeled PLF1 and successfully competed it off using 10 mM mannose 6-phosphate, the MRP/PLF mRNA could not be detected using *in situ* hybridization on mid-sagittal sections of gestational day 16 embryos or RT-PCR on gestational day 16 embryonic spine RNA preparation (25). To determine the expression time course and isoform of *mrp/plf* mRNA expressed in the embryonic spine from day 13 to day 19 of gestation, RT-PCR and Northern blot analysis were performed. No *mrp/plf* mRNA was detected by Northern blot on total RNA isolated from whole embryos aged from day 9 to day 19 of gestation (data not shown). Nor was *mrp/plf* mRNA detected by RT-PCR in the spine, tail, skin, heart or ribs collected from gestational day 18 embryos. However, a positive control made of day 11 placenta total RNA showed that *mrp/plf* mRNA was detectable by both methods. In addition, GPDH mRNA used as a loading control for the Northern blot and as a positive control for the RT-PCR showed that the total RNA isolated contained intact mRNAs (data not shown).

DISCUSSION:

Examination of uterus weight and total DNA both showed a rapid increase from day 9 to 14. These two events correlate to the time when the uterus undergoes decidualization which is characterized by uterine stromal cell proliferation and differentiation as well as a massive infiltration of leukocytes (19, 20) and increased vascular permeability (21). The
combination of these four events can account for the rapid increase in uterine wet weight observed from gestational day 9 to 14. Differentiating stromal cells replicating their DNA without cell division (enderoreplication), cell proliferation of other stromal cells, and leukocyte infiltration can all account for the rapid increase in uterine DNA. During gestation, the decidua is degraded to make room for the growing embryo. Up to day 14, the embryo wet weight increases very slowly, which is paralleled by a slow degradation of the decidua (38). The decidual degradation, accomplished by slow resorption of the decidual cells, accentuates the rapid increase in uterine wet weight and DNA amounts.

From day 14 to birth, DNA content in the uterus does not increase significantly whereas the uterine wet weight still increases. Interestingly, during this period the decidua is rapidly degraded to accommodate the now rapidly growing embryo (38, 39). The increase in wet weight without apparent increase in DNA synthesis suggests that during late gestation there is a combination of increase in uterine cell size (hyperplasia) and increase in protein accumulation or retention of water by the uterus. From day 14 to day 16 there is no increase in wet weight. This would account for the switch between the end of the decidual regression and the DNA synthesis-independent increase in wet weight of the uterus.

The plasma profiles of both estradiol-17β and progesterone measured here are very similar to the one obtained by McCormack and Greenwald (17). Estradiol-17β is present in the uterus and the placenta, and peaks on two days. Gestational day 6 is the highest with the smallest peak at gestational day 14. The uterus and placenta possess the enzymes necessary for estradiol-17β synthesis (40, 41) suggesting that these tissues can synthesize estradiol-17β. However, it can not be ruled out that the high levels detected at gestational day 6 and the late
peak at gestational day 14 are due to the accumulation of estradiol-17β in the placenta and the uterus rather than *de novo* synthesis. Furthermore, the significance of the high levels of estradiol-17β in the placenta and uterus at day 6 is not known. The peak observed at day 14 correlates with rapid embryo growth and rapid cell death in the decidua. The biology of the decidua and the placenta during this time in pregnancy is not well studied. Therefore it is difficult to speculate on the relevance and the role for the presence of estradiol-17β in the uterus and the placenta at day 14.

In the placenta and the uterus the levels of progesterone are low at the beginning of gestation and peak at day 10 to rapidly decrease to their minimum after day 14. In the placenta, all the enzymes needed for progesterone synthesis from cholesterol are present in the trophoblastic giant cells and their gene expression patterns during gestation are identical to the pattern of progesterone accumulation in the placenta (41). In addition, progesterone secretion in cultured mouse trophoblast cells of different gestational ages, showed a profile of expression identical to the one observed for the whole placenta (42). Progesterone has been shown to decrease mouse placental lactogen I (mPL-I) expression in placental mixed primary cell cultures (43). The luteotropic action of mPL-I is thought to be replaced in late gestation by mouse placental lactogen II (mPL-II) which starts to be detected in the circulation in mid-gestation, and stays at high concentration throughout the rest of gestation. Progesterone could be involved in this switch by lowering the amounts of mPL-I and increasing the amounts of mPL-II. The reason for the switch in the mPL is unclear as the same trophoblastic giant cells secrete both placental lactogens (44).
Unlike the effect of progesterone on mPL-I and mPL-II secretion, progesterone had no effect on the accumulation of MRP/PLFs in the conditioned medium of minced placental tissue cultures from day 9 of gestation. Similarly, estradiol-17β did not change the accumulation of MRP/PLFs in the conditioned medium of the minced placental tissue cultures from day 9 of gestation. At day 14 of gestation, the decrease of MRP/PLF expression is accentuated, which correlates with the presence of estradiol-17β in the uterus and the placenta at day 14. Estradiol-17β could play a role in down regulation MRP/PLF expression in the placenta, similarly to the action of progesterone on mPL-I around day 10 of gestation.

The day 11 minced uterine organ culture and uterine primary cell cultures were used to determine if the recombinant His-MRP/PLFs could stimulate DNA synthesis through the uterine receptor in vivo. His-PLF1 did not stimulate $^3$H-thymidine incorporation in the day 11 minced uterine organ cultures. A similar experiment was done with MRP3 after enterokinase removal of the histidine tag and no increase in $^3$H-thymidine incorporation was observed in the day 11 uterine organ cultures. His-PLF1, His-MRP3 were tested on day 11 uterine primary cell cultures prepared using both methods described in Materials and Methods. No significant $^3$H-thymidine incorporation was detected after stimulation of the cells isolated by either method with the different recombinant MRP/PLFs. One conclusion from these experiments is that recombinant MRP/PLF isolated from the conditioned medium of stably transfected 293 cell could not bind to the uterine cell receptors. Another conclusion is that in 293 cells the isolated recombinant MRP/PLFs were biologically active, but the minced uterine organ cultures or the primary uterine cell cultures could not give the previously detected
To avoid the variables introduced by cell culture, the day 11 uterine membrane binding assay (4) was used to test the binding of His-MRP3 and MRP3, with the histidine tag removed, to the uterine MRP/PLF receptor. No specific binding of $^{125}\text{I}$-His MRP3 or $^{125}\text{I}$-MRP3 was detected on day 11 uterine membranes. This suggests that the 293 cell recombinant MRP3, with or without histidine tag, does not bind to the uterine receptor. Recombinant PLF1 from CHO cells was shown to bind to the IGFII/M6P receptor present on day 16 maternal liver membranes (23). When tested, $^{125}\text{I}$-His MRP3 or $^{125}\text{I}$-MRP3 did not bind specifically to the day 16 liver membrane via the IGFII/M6P. However, r-IGF-II bound specifically to day 16 liver membrane suggesting that the IGFII/M6P was present on the day 16 liver membrane tested and that the binding assay was performed properly.

In the receptor binding assay, it was found that the type of filters used to separate the bound radioactive proteins from the free form is important. The glass fibers filters from Whatman, used by Lee et al. (23), retained more radioactivity compared to the PVDF filters from Millipore, used by Nelson et al. (4), suggesting that more membrane vesicles were retained which ultimately led to better differentiation between experimental conditions.

The glycosylation patterns of MRP/PLFs could have a serious impact on the binding activity of MRP/PLFs to their receptors. Analyses of the glycosyl groups on recombinant glycoproteins expressed by CHO cells, 293 cells and SF9 insect cells, showed that these cells secreted glycoproteins with different sugar residue composition (45, 46). Using the overlay assay developed by Valenzano et al. (32), at least 50 % of the His-MRP3 isolated from
293 cells had mannose 6-phosphate present. Interestingly, the His-MRP3 that did not bind the IGFII/M6P receptor in the overlay assay had a slower relative migration on the non-reducing SDS page. This slower migrating band disappears when β-mercaptoethanol is added suggesting that the three possible disulfide bridges present in native MRP/PLF changes the conformation of MRP/PLF to allow different migrating characteristics in the reducing SDS-PAGE. Migration could be independent of the tertiary structure but relies on the differences in charges produced by the absence of mannose 6-phosphate, or the presence of other sugars.

The presence of MRP/PLF in the embryo was further investigated to determine if the MRP/PLF protein detected in the spine and heart structures of day 10 to day 18 embryo endothelial cells by binding to the M6P/IGFII receptor and by immunohistochemistry (25) was due to localized synthesis or export from the placenta. Dudley reported the presence of mrp/plf mRNA by RT-PCR and Northern blot analysis in day 11 heart and tail as well as gestational day 17 embryonic skin, heart, spine, rib (24). Northern blots performed on whole embryos from gestational day 9 to day 19 did not detect mrp/plf mRNA except for a very light band in day 10 embryo which is likely to be due to placental tissue contamination during the dissection procedure. In effect, the embryo is very small and looks very similar to placental tissue and day 10 placenta has the highest amount of mrp/plf mRNA during all of gestation. Day 11 embryos which are easier to separate from the placenta did not show any mrp/plf mRNA. RT-PCR and Northern blot performed on day 18 embryonic tissues (skin, heart, spine, rib, tail) as well as isolated embryonic spines (day 13 to day 19 of gestation) did not show the presence of any mrp/plf mRNA. These results support the data published by Jackson and Linzer (25) showing that no mrp/plf mRNA could be detected by in situ hybridization and
RT-PCR in the spines and hearts of embryos. This leads to the conclusion that the only known cells secreting MRP/PLFs are the placental trophoblastic giant cells (1) and keratinocytes (47). The binding of MRP/PLF to the embryo parallels the localization of the IGFII/M6P receptor in the embryo (48) suggesting that the role played by MRP/PLF in the embryo is via the IGFII/M6P receptor.

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CHAPTER 4.

EXPRESSION OF 24p3/UTEROCALIN, A LIPOCALIN FAMILY MEMBER, IN THE EMBRYO, JUVENILE AND ADULT MICE

ABSTRACT:

The secreted protein, 24p3/uterocalin is one of several proteins superinduced upon the stimulation of quiescent 3T3 cells with fibroblast growth factor. Expression of 24p3/uterocalin was detected in the embryonic spine on gestational day 13, shown to increase throughout gestation and continued to increase over the life time of the mouse. In the liver 24p3/uterocalin mRNA levels were the highest in the late-gestational fetal liver and decreased within the first week after birth. The 24p3/uterocalin mRNA was also detected in the spleen of juvenile adults. Several types of tissue undergoing remodeling show an increase in 24p3/uterocalin suggesting a role in tissue remodeling. The presence of 24p3/uterocalin mRNA in the fetal and juvenile liver and in the spine after birth appeared to correlate with hematopoiesis in these tissues and therefore it is hypothesized that 24p3/uterocalin may have a potential role in events related to both embryonic and adult hematopoiesis.

INTRODUCTION:

Mitogens such as fibroblast growth factor (FGF), epidermal growth factor (EGF) or serum, stimulate the secretion of several proteins in cell culture. One of these proteins is mitogen regulated protein/proliferin (MRP/PLF) whose secretion from quiescent 3T3 cells is induced 20 hours after the addition of any of the mitogens mentioned above (1). Similarly,
SIP24 was identified as a protein secreted after FGF treatment of quiescent mouse 3T3 cells. This SIP24 induction in response to mitogen stimulation occurred within 10 hours of FGF addition to these 3T3 cells. Addition of the protein synthesis inhibitor cycloheximide with the FGF increased SIP24 accumulation 4 to 5 fold in the conditioned medium of quiescent 3T3 cells. Unlike SIP24, MRP/PLF was not superinduced by the addition of cycloheximide with FGF. This observation suggests that although MRP/PLF and SIP24 can both be stimulated by FGF, their synthesis and secretion appear to be regulated differently (2).

The mRNA 24p3 was up-regulated in mouse primary kidney cell cultures infected with the polyoma or SV40 viruses (3). The 24p3 protein was also found to be a major secreted protein when PU5.1.8 macrophage cells were stimulated with LPS (4). Protein sequencing of peptides obtained from enzymatic digestion of SIP24 revealed that it was identical to the 24p3 protein (5). Careful analysis of the amino acid sequence of 24p3 revealed that it belonged to the growing superfamily of lipocalins (6, 7). The lipocalin family is characterized by a low amino acid alignment (less than 20 %), but a high degree of conservation in 3 domains and a common folding pattern. The so-called lipocalin fold is a hydrophobic cavity formed by 8 antiparallel β-sheets allowing the binding of hydrophobic molecules such as retinoids, fatty acids, and steroids (8).

Studies on the regulation of the 24p3 gene expression led to the cloning of the 24p3 promoter and gene. Two, overlapping, glucocorticoid response element consensus sequences were found on this promoter suggesting a possible regulation by glucocorticoids (9). Subsequently, addition of the glucocorticoid, dexamethasone, to mouse embryonic fibroblast L cells was shown to increase the mRNA, as well as levels of secreted 24p3 protein levels (9).
Concurrently, Liu et al. showed that the addition of dexamethasone with cycloheximide to mouse 3T3 and BNL (embryonic liver) cells, increased 24p3 protein levels (5). Injection of dexamethasone in both male and female adult mouse triggered the accumulation of 24p3 mRNA in the liver. In addition, during acute phase response triggered by turpentine injection, 24p3 mRNA levels increased in the mouse liver as well as in the uterus. This led to the conclusion that 24p3 was a new, positive acute phase, response protein (5).

During pregnancy, the uterus at gestational day 11 contains detectable amounts of 24p3 mRNA (5). At birth, mRNA levels of 24p3 increase in the uterus to be 16 fold higher the day after birth than in the non-pregnant uterus. The levels then decrease rapidly 2 to 3 days after birth as the uterus undergoes regression (10, 11). The 24p3 protein levels in the uterus follow closely the pattern of expression of 24p3, and in situ immunohistochemistry suggested that 24p3 was located in the luminal and glandular epithelium. The high expression levels of the lipocalin 24p3 in the uterus led to the renaming of 24p3 as uterocalin (11).

Upon injection of the estrogen analogue, diethylstilbesterol, 24p3/uterocalin secretion in the uterine luminal fluid was stimulated in immature female mice (12). A more detailed analysis in mature mice revealed that 24p3/uterocalin mRNA and protein was expressed at high levels before ovulation, when high amounts of estradiol are present in the plasma. After ovulation, progesterone increases and the amounts of 24p3 mRNA and protein were shown to decrease. This ovarian steroid model could be replicated in immature female mice injected with estradiol and/or progesterone. These observations suggested that 24p3/uterocalin could be regulated directly or indirectly by ovarian steroids (13).
Two surveys of the tissue specific expression patterns for 24p3/uterocalin revealed expression in several organs. Chu et al. looked in 42 to 56 day-old male and female mice and found mRNA expression in the lung, spleen, vagina and epididymis. Other tissues like the brain, thymus, heart, liver, kidney, pancreas, ovary, testis, prostate did not express detectable levels of 24p3/uterocalin mRNA (12). Garay-Rojas et al. using younger, 21 day-old male mice found 24p3/uterocalin mRNA expression in the lung, spleen, testis, and liver. Tissues studied that did not express detectable levels of mRNA were brain, thymus, kidney and muscle. Further study of 24p3/uterocalin expression in day 10 mice revealed that in the liver, spleen and kidney the mRNA was expressed at higher levels compared to 21-day old mice. The 24p3/uterocalin mRNA levels decreased to undetectable levels in the kidney by day 25 and in the spleen and liver at 45 to 65 days, respectively (9).

Interestingly, in vitro studies on the effect of interleukin-3 (IL-3) deprivation on the mouse leukocytic cell line FL5.12 demonstrated an upregulation of 24p3/uterocalin mRNA and protein secretion in FL5.12 cells undergoing apoptosis in response to the removal of IL-3. These studies showed that the secreted 24p3/uterocalin triggered apoptosis via an autocrine regulatory loop (14).

Additional proposed functions for 24p3/uterocalin include involvement in tissue remodeling. After weaning, the mammary gland undergoes involution which is accompanied by rapid tissue resorption. The expression profile in the mammary gland of 24p3/uterocalin mRNA and protein correlates with the regression of this tissue (15). Also, during endochondral bone formation, chondrocyte differentiation is accompanied by upregulation of the chicken and rat homologues of 24p3/uterocalin (16). The 24p3/uterocalin human
homolog, Neutrophil gelatinase-associated lipocalin (NGAL), is expressed by leukocytic cells in the bone marrow and in particular by the neutrophils (17). The survey of several human tissues showed varied expression in tissues like the bone marrow, thymus, liver, fetal spleen, mammary gland, trachea, lung, spleen (18). No definitive role for 24p3/uterocalin and its homologs in the human, rat and chicken can be deduced from this varied expression pattern throughout the body.

Many putative functions have been proposed for 24p3/uterocalin and homologs, yet no specific roles have been clearly defined. To provide additional insight into the function of 24p3/uterocalin, a study of its mRNA expression in liver, spleen and spine during mouse embryogenesis was undertaken.

MATERIALS AND METHODS:

Animals and reagents

Female CF1 mice, originally obtained from Charles River Laboratories, Inc., were maintained as a breeding colony for the Nilsen-Hamilton laboratory in one of the Iowa State University animal facilities, with a 12 h light, 12 h dark cycle and food and water ad libitum. All animals were housed and treated according to current NIH guidelines. The research reported here was conducted in accordance with the standards set forth in the NIH Guide for the Care and Use of Laboratory Animals. Prior approval was obtained from the Iowa State University Committee on Animal Care for all procedures performed on the animals used in these studies. Animals were killed by cervical dislocation or CO₂ asphyxiation before removal.
of tissues for the described studies. The detection of a copulation plug or sperm in the vaginal smear was used to determine day 0 of gestation.

**RNA isolation, Northern Blotting and RT-PCR**

Total RNA was isolated from 50 to 100 mg of frozen tissue using TRIzol® reagent (Gibco, Invitrogen Corporation) and resuspended in RNase free water. Total RNA, 15 to 30 μg determined by absorption at 260 nm, was dried using a vacuum centrifuge (SpeedVac, Savant) and the pellet was resuspended in 15 μl of RNA loading buffer (48 % formamide, 0.11 M MOPS, 6.4 % formaldehyde, 5.3 % glycerol, 0.25 % bromophenol blue). The samples were boiled 2 min and loaded onto a 1 % agarose gel, containing 1.7 % formaldehyde, 0.5 μg/ml ethidium bromide. Electrophoretic separation was completed at 60 V for 3 h in 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA pH 7.0. The gel was rinsed at room temperature 15 min in deionized water followed by 15 min in 50 mM NaOH. The RNA was transferred to Zeta-Probe® GT Blotting membrane (Bio-Rad) by alkaline capillary transfer overnight in 50 mM NaOH. The membrane was washed 10 min with 2X SSC (1 X SSC: 0.15 M NaCl, 15 mM sodium citrate pH 7.0) and baked 15 min at 80 °C under vacuum. Before probing, the membrane was pre-hybridized at 47 °C for 6 h with 100 μg/ml yeast tRNA and salmon sperm DNA, boiled 2 min and chilled on ice, in 0.6 M NaCl, 40 mM NaH_2PO_4, 50 mM EDTA, 7 % SDS, 1 % PEG 20000, 40 % formamide, 0.1 % NP40, pH 7.4. A ^32^P-labeled 24p3/uterocalin probe was generated by using the Multiprime DNA labeling system (Amersham Pharmacia), 24p3/uterocalin cDNA and 3000Ci/mmol α-^32^P-dCTP (ICN). Unincorporated dNTPs were separated from the labeled probe by gel filtration using a
micro bio-spin® P-30 Tris chromatography column (Bio-Rad). The probe in 10 mM Tris pH 7.4 was boiled 5 min and chilled on ice before adding to the blot in the pre-hybridization solution for a 16 h incubation at 47 °C. The membrane was washed twice for 15 min with 0.5X SSC, 0.1 % SDS at room temperature followed by 2 washes for 15 min with 0.2X SSC, 0.1 % SDS at 47 °C. The membrane was dried and exposed to a PhosphorImager screen at room temperature. The screen was scanned and quantitated using a PhosphorImager 400A and ImageQuant 3.3 software (Molecular Dynamics).

The “house-keeping” gene glyceraldehyde-3-phosphate dehydrogenase (gpdh) was used to normalize RNA loading. Before reprobing, the 32P-24p3/uterocalin probe was removed from the membrane by boiling the membrane twice for 3 min in 0.1 X SSC, 0.1 % SDS. The striped membrane was pre-hybridized, hybridized with 32P-labeled gpdh probe, washed and exposed to a PhosphorImager screen as described above.

RT-PCR using the SuperScript™ One-Step RT-PCR kit from Invitrogen Life Technologies was performed on 1 μg of total RNA. Uterocalin cDNAs were amplified using 0.2 μM of the primers QL921 (5' GGGGGCTCGAGAGACCTAGTAGCTGTGGAAA 3') and QL920 ( 5' GGGGGAAGCTTGGGGGCATGTATTTATTCAG 3'). Gpdh cDNA was amplified using 0.2 μM of the primers GPDH5' (5' TGT GGATGGCCCCTCTGGAAA 3') and GPDH3' (5' GTTTCTTACTTCCTTGGAGGC 3'). The cDNA synthesis was done at 50 °C for 30 min followed by a pre-denaturation step at 94 °C for 2 min. The PCR amplification consisted of 40 cycles (94 °C for 15 s, 60 °C for 30 s, 72 °C for 50 s) and a final extension step at 72 °C for 10 min using the DNA engine Opticon (MJ Research). The RT-PCR
products were resolved on a 1.5 % agarose gel, containing 0.5 µg/ml ethidium bromide using TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA pH 8.5) at 80 V for 1 h.

**Immunohistochemistry**

Embryos at different days of gestation were dissected and rinsed in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). The embryos were then fixed in 2 % paraformaldehyde in 140 mM NaCl, 20 mM sodium phosphate pH 7.0 for a minimum of 12 h. The embryos were then processed for paraffin embedding and 5 µm sections were prepared by the Histopathology Laboratory at the Department of Veterinary Pathology (Iowa State University).

The slides were warmed at 60 °C for 30 min using a slide warmer (Chicago Surgical and Electric Co.). All subsequent steps were performed at room temperature using Vectastain Elite® ABC (Vector). The melted paraffin was removed by incubating twice in xylene for 3 min each followed by 3 min in 100 % ethanol. The tissue were rehydrated by incubation for 3 min in 95 % ethanol followed by 3 min in 70% ethanol and finally 3 min in glass distilled water. The sections were incubated in 3 % hydrogen peroxide, 10 % methanol in PBS and washed twice (5 min each) in PBS twice. The slides were blocked in blocking solution (1:70 normal goat, 0.2% bovine serum albumin (immunohistochemical grade, Vector), in PBS) for 30 min. The blocking solution was removed and replaced with fresh blocking solution containing rabbit antiserum 87Rb07e against 24p3/uterocalin or the pre-immune rabbit serum at a final dilution of 1:500 or 1:700. After incubation for 1 h, the slides were washed. The sections were then incubated with biotinylated anti-rabbit IgG in blocking
solution (1:100) for 30 min. After washing the sections twice (5 min each) in PBS, the ABC reagent (pre-incubated 30 min) was added for 30 min. The sections were washed twice (5 min each) in PBS, and DAB substrate, prepared according to manufacturer’s instructions (Vector), was added for 7 min. After washing twice (5 min each) in glass distilled water, the sections were counterstained for 10 min using Weigert’s Iron hematoxylin stain (19). The slides were rinsed with glass distilled water, differentiated in acid alcohol (1 % v/v HCl in 70 % ethanol), rinsed until bright blue in ammonia water (0.2 % v/v ammonium hydroxide in water) and washed under running deionized tap water for 10 min. The tissue were dehydrated by incubating for 3 min in the following solutions: 70 %, then 95 %, then 100 % ethanol, and twice in xylene. Coverslips were mounted on the tissue sections with Permount (Fisher).

RESULTS:

Detection of 24p3/uterocalin mRNA in embryonic spine by RT-PCR

The rat homolog of 24p3/uterocalin is expressed during endochondral bone formation in gestational day 17 tibia (16). The spine also undergoes endochondral ossification (20) during the formation of the vertebrate bone in the mouse embryo. In order to determine whether the spine could express 24p3/uterocalin, the mRNA presence in embryonic spine was examined by RT-PCR. 24p3/uterocalin mRNA was detected in spine isolated from embryos from days 13 to 19 of gestation (Figure 1).
Figure 1: 24p3/uterocalin detection by RT-PCR on embryonic spine.

RT-PCR was performed on 1 µg of total RNA isolated from spines of embryos collected at day 13 (e13), day 15 (e15), day 17 (e17) and day 19 (e19) of gestation. The positive control for 24p3/uterocalin (24p3 +Cont.) was obtained by RT-PCR using 1 µg of total RNA isolated from a day 1 post-partum uterus. For gpdh amplification, the primers GPDH5' and GPDH3' were used. For 24p3/uterocalin amplification, the primers QL921 and QL922 were used. The amplified cDNA were electrophoresed on a 1.5 % agarose gel.

24p3/uterocalin mRNA expression in embryo, newborn, juvenile and adult mice

To compare the levels of 24p3/uterocalin mRNA expressed during the period from late-gestation embryo to adult female mouse, data was gathered for the spine, liver, and spleen. During development, these tissues are involved in the formation and regulation of blood cell lineages. All three tissues investigated expressed 24p3/uterocalin mRNA at one point during the life of the mouse. The embryonic liver expressed the highest amounts of 24p3/uterocalin mRNA when compared to the other two embryonic tissues (Figure 2). The highest amount of 24p3/uterocalin in the liver was found during late embryonic development and decreased after birth to be undetectable in 3 week old mouse. In the spleen, the juvenile
mouse had high levels of 24p3/uterocalin mRNA on days 8 and 21 of age. However, spleenic 24p3/uterocalin mRNA levels decreased after day 49. In the spine, the levels of 24p3/uterocalin mRNA increased throughout gestation and then over the life time of the mouse (Figure 3). The levels of 24p3/uterocalin mRNA in the 49 day-old mice were probably underestimated due to a low quantity of total RNA applied in these lanes (Figure 2A). The levels of 24p3/uterocalin mRNA in the tail during gestation and over the life time of the mouse were also examined, but no significant levels of expression were detected (Figure 2).

Figure 2: Expression of 24p3/uterocalin in murine spine, liver, spleen and tail during aging.
Panel A: Total RNA from spine, Panel B: Total RNA from liver, Panel C: Total RNA from spleen, Panel D: Total RNA from tail. For each panel, 30 μg of total RNA isolated from each tissues were resolved on an agarose gel and stained with ethidium bromide to determine the relative amounts of 28S and 18S rRNA for equal loading (first panel). The positive control for 24p3/uterocalin (+ control) was 5 μg of total RNA from a day 1 postpartum uterus. After transfer, the blots were all treated as one. 24p3/uterocalin was detected by blot exposure and detection via a PhosphorImager screen. After developing the screen, the blot was stripped off the 24p3/uterocalin probe and gpdh was detected by blot exposure and detection via a PhosphorImager screen. The days at which the tissues were collected are gestational day 17 (e17), day 18 (e18), day 19 (e19) for embryonic tissues. For juvenile and adult female mice the tissues were collected at 1 day (d1), 8 days (d8), 21 days (d21), 49 days (d49), 116 days (d116) after birth.
Figure 3: mRNA quantitation in murine spine, liver and spleen.

The intensity of the bands from the Northern blots shown in Figure 2 were quantitated (ImageQuant software). The quantitative data of 3 other blots were added to determine the profile of expression in the embryonic spine. Ratio of the counts present in 24p3 band over the counts present in the gpdh band was used to normalized the data, and the value of the ratio was arbitrarily assigned to 1 for d1 liver and spine and for d8 spleen. The error bars represent +/- one standard deviation. The embryonic stage at which the tissues were collected are represented as e13, e15, e17, e18, e19 for embryonic tissues collected at gestational days 13, 15, 17, 18, 19 respectively. For juvenile and adult female mice, the age at which the tissues were collected is represented as d1, d8, d21, d49, d116 for tissues collected at 1 day, 8 days, 21 days, 49 days, 116 days after birth. The absence of bar indicates that tissues were not examined on that given day. The solid lines above the graph represent the time when hematopoiesis takes place, the dashed line shows the presence of hematopoiesis but the organ is not the major location of hematopoiesis at that time.
Detection of uterocalin protein by immunohistochemistry

To determine if the 24p3/uterocalin protein could be detected in the tissues where the mRNA was detected, immunohistochemistry was performed on mid-sagittal section of embryos from gestational day 11 to day 19.

No specific protein signal was observed on mid-sagittal sections of any of the embryos examined. A cross-section of a day 1 postpartum uterus was used as a positive control and showed an expected pattern of staining in the luminal and glandular epithelium (data not shown). The antiserum background staining was minimal. The absence of signal suggested that the local protein concentration was undetectable by immunohistochemistry, or 24p3/uterocalin was secreted to another location.

DISCUSSION:

The study of 24p3/uterocalin mRNA expression in the liver, spleen and spine provides additional insight on the location of 24p3/uterocalin expression during mouse embryogenesis as well as during aging. The 24p3/uterocalin mRNA profile in the spleen of female mice was similar to the profile previously reported in male mice (9). In addition, detectable levels of 24p3/uterocalin mRNA were observed as late as 116 day old in female. No major transitions in the role or anatomy of the spleen occur between day 21 and 49 when the levels of 24p3/uterocalin drop dramatically. However, mice are weaned around day 21 and become sexually mature around day 35. A more detail analysis of the 24p3/uterocalin mRNA levels between day 21 and 45 in male versus female mice could suggest a function of the spleen which is related to weaning or sexual maturation.
The high levels of 24p3/uterocalin in day 8 female mouse liver rapidly decreased to be at their lowest level at day 21. The profile of 24p3/uterocalin mRNA in the liver of 10 day old and adult male mice showed high expression. The mRNA plateaued by day 45 and declined to undetectable limits by 75 days in the adult mouse (9). This difference in the 24p3/uterocalin profile in the young and adult mouse is unclear but could be related to the sex or strain of the animals used in each study. The determination of the mRNA profile in the embryonic and the newborn mouse liver adds new insight to possible roles of 24p3/uterocalin during gestation. The period of expression in the liver corresponds to the period when hematopoiesis actually takes place in the liver before its slow and gradual transition to the permanent localization of hematopoiesis to the bone marrow at gestational day 15 (21). Before determining a function for 24p3/uterocalin in the liver, it will be critical to determine the cell type expressing the mRNA. 24p3/uterocalin might be involved in the transition of hematopoiesis from the liver to the bone marrow by the removal of specific cells from the liver via apoptosis. This role is suggested by the induction of apoptosis in proB-lymphocytes, cells involved in hematopoiesis, by 24p3/uterocalin (14). Conversely, the proposed protective role of 24p3/uterocalin during the acute phase response and tissue involution could be involved in protecting fetal liver from degradation by leukocytes present during hematopoiesis in the fetal liver.

Most interestingly, 24p3/uterocalin levels in the spine increased as the animal aged. The rapid increase between gestational day 19 and day 1 newborn mice correlates with the observation that transition of hematopoiesis to the bone marrow intensifies around birth (22). The levels of 24p3/uterocalin were the highest in the 116 day old spine. It is not clear why as the animal ages, the level of 24p3/uterocalin increases. The continued increase in
24p3/uterocalin message as the animal ages, could also be linked to the way total RNA was collected. Whole spines were used to isolate RNA until the animals were 8 days old. After 21 days of age, only the thoracic region was used to isolate RNA. The undetectable levels of mRNA in the tail suggests that the thoracic portion of the spine contains most of the 24p3/uterocalin mRNA. Thus, it is possible that the total RNA population was enriched with 24p3/uterocalin mRNA in the older mice compared to the embryonic and younger mice. Interestingly, the bone marrow in the spine of the thoracic region is where most of hematopoiesis takes place (23).

The data described here, along with the correlation of 24p3/uterocalin expression under conditions associated with apoptosis and tissue remodeling allows for the formation of a hypothesis that would implicate a role for 24p3/uterocalin in hematopoiesis, particularly during embryogenesis.

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CHAPTER 5.

GENERAL CONCLUSIONS

MRP/PLFs are members of the prolactin-growth hormone family. They are glycoproteins secreted by the placenta during mid-gestation and are thought to play a role in both angiogenesis during neovascularization of the uterus and in stimulation of uterine cell proliferation. To gain insight into the role of MRP/PLFs in the uterus during mid-gestation, I became interested in studying the interactions between MRP/PLFs and its uterine receptor(s) as well as the role played by the carbohydrate moieties of MRP/PLFs in mediating the ability of MRP/PLFs to bind to the uterine receptor. I also was interested in studying the transduction pathway(s) of MRP/PLFs after activation of the uterine receptor.

To begin studying the roles of MRP/PLFs during mid-gestation, some of the physiological changes occurring in the utero-placental compartment were characterized throughout gestation. Examination of the uterus during the course of gestation demonstrated a parallel increase in wet weight and in DNA content. This increase is correlated with the peak of secretion of MRP/PLFs by the placenta from gestation days 11 to 14 and supports the hypothesis that MRP/PLFs are involved in uterine maintenance and development.

To understand the glycoprotein-ligand interaction with the uterine receptor, cDNA expression vectors were created for the different MRP/PLF family members. All four known mrp/plf cDNAs were modified to include a removable N-terminal histidine tag. Recombinant proteins were successfully expressed and purified from the conditioned medium of stably transfected human 293 cells. Experiments were designed to examine the ability of the
recombinant MRP/PLF family members to bind uterine membranes and stimulate DNA synthesis in uterine cells. The results obtained and summarized in Chapter 3 were significantly different to those previously described (1), (Rosenzweig, unpublished data).

The absence of $^3$H-thymidine incorporation in minced uterine organ and primary uterine cell cultures upon addition of recombinant MRP3 (with or without the histidine tag) suggested that recombinant MRP/PLF secreted by 293 cells was non-active (Chapter 2). However, several difficulties were encountered with both minced uterine organ and primary uterine cell cultures. In the uterine organ cultures, Rosenzweig observed a 2 to 3 fold increase in thymidine incorporation upon the addition of MRP/PLF purified from BN/L cells. In general, previous reports (Rosenzweig, unpublished data) and my observations showed that uterine organ culture incorporated low numbers of TCA precipitable counts with high variability which is incompatible with the small increases in $^3$H-thymidine incorporation.

The increase in $^3$H-thymidine incorporation by quiescent Swiss 3T3 cells stimulated by untreated serum (data not shown) or primary keratinocytes stimulated with KGF (Data not shown) showed that $^3$H-thymidine incorporation can be detected in different cell types attached to coverslips. Data from Rosenzweig, showed that MRP/PLF isolated from BN/L cells gave a 4 to 5 fold increase in $^3$H-thymidine incorporation in primary uterine cell culture isolated by the method described in Nelson et al. (1). The optimum conditions for $^3$H-thymidine incorporation were observed when day 11 uterine primary cell cultures were treated with 5 ng/ml MRP/PLF for 30 hours followed by a 4 hour pulse with 8 µCi/ml $^3$H-thymidine (Nilsen-Hamilton lab, unpublished data).
However, when primary uterine cell cultures were isolated for an independent repeat of the $^3$H-thymidine incorporation assay, these cells did not have the growth characteristics observed when previously prepared by Rosenzweig. Different batches of fetal calf serum were tested to regain the original cell growth characteristics. In addition, techniques for cell isolation and cell attachment were modified to reestablish the cell growth characteristics observed by Rosenzweig. McCormack et al. described a method to isolate different rat uterine cell types. This method was used to isolate day 11 mouse uterine stromal and epithelial cells. The mouse uterine cells isolated by this method had similar growth and morphological characteristics to the one reported by McCormack et al. (2). After re-establishing the day 11 uterine cell cultures, several MRP/PLF preparations purified from Swiss 3T3 cells (3), BN/L cells (1), baculovirus (Nilsen-Hamilton lab, unpublished results) and more recently recombinant His-PLF1, His-MRP3 and MRP3 without the histidine tag (Chapter 2) were tested for their ability to stimulate $^3$H-thymidine incorporation. However, no significant incorporation was observed when the different uterine cell types were treated with the various MRP/PLF batches as mentioned above.

Interestingly, after examination of the cell morphology, the cells incorporating high tritium counts had a different morphology as compared to the cells associated with low tritium counts. A time course of basal $^3$H-thymidine incorporation compared with the associated morphology of non-stimulated day 11 uterine cells showed that a switch in morphology is associated with a rapid drop in $^3$H-thymidine incorporation (data not shown). This suggests that without the addition of any growth factors, the uterine cells in culture underwent
differentiation and decreased their DNA synthesis. If one of the two cell types is unresponsive to MRP/PLFs this could account for the high variation in counts observed in the assay.

In conclusion, the lack of $^3$H-thymidine incorporation in the minced uterine organ and primary uterine cell cultures upon stimulation with MRP/PLF, isolated from several cell types, suggests the presence of contaminant(s) in the protein preparations. The contaminant(s) present in the MRP/PLFs isolated from Swiss 3T3 cells, BN/L cells, baculovirus, or 293 cells inhibited the $^3$H-thymidine incorporation of the uterine cells. Conversely, contaminant(s) present in the original BN/L preparation used in Nelson et al. (1) increased the $^3$H-thymidine incorporation of the uterine cells.

The receptor binding activity of His-MRP3 was tested on isolated membrane preparations. This method decreases the number of parameters associated with tissue culture, like cell type and serum lot, for example. MRP3 with or without its histidine tag did not bind specifically to day 16 maternal liver (4) or day 11 uterine (1) membrane preparations. The absence of binding to these liver membranes suggested that His-MRP3 or MRP3 isolated from 293 cells was unable to bind to the IGF-II/mannose-6-phosphate (IGFII/M6P) receptor. The presence of the IGF-II/M6P receptor on day 16 liver membrane preparations was confirmed with the successful binding of IGF-II. Furthermore, presence of mannose 6-phosphate on the His-MRP3 was demonstrated in an overlay assay using the bovine IGF-II/M6P receptor. Interestingly, in the overlay assay only 50 % of the His-MRP3 could be bound by the bovine IGF-II/M6P receptor, indicating either that 293 cells do not add mannose 6-phosphate efficiently or the glycosidic residues were dephosphorylated subsequent to secretion. High affinity binding of glycoproteins to the IGF-II/M6P receptor requires the presence of several
mannose 6-phosphates on the same glycoprotein to allow efficient dimerization of the receptor (5). It is possible that in the overlay assay the highly localized concentration of His-MRP3 on the nitrocellulose membrane, allowed for an artificial concentration of mannose 6-phosphate residues enabling high affinity binding of the IGF-II/M6P receptor. In solution, this localized concentration of mannose 6-phosphate would not occur and the low affinity of the IGFII/M6P receptor for His-MRP3 resulted in no detectable binding activity in the day 16 liver membrane binding assay.

The source of the protein utilized in the day 16 liver membrane assay by Lee and Nathans was recombinant PLF1 from CHO cells, whereas the protein used in Chapter 3 was recombinant MRP3 (different from PLF1 by 2 amino acids) purified from 293 cells. PLF1 and MRP3 have the same number of putative N-glycosylation recognition sequences indicating that they can potentially be glycosylated identically. Therefore if glycosylated properly, PLF1 and MRP3 should both bind to the IGFII/M6P receptor which is mainly mediated by the carbohydrate moiety rather than the protein moiety of glycoproteins (6-10). However, reports are suggesting that CHO cells and 293 cells generate different glycosylation patterns (11, 12). It is then possible that the 293 cell glycosylated-MRP3 has a glycosylation pattern such that it has low binding affinity for the IGFII/M6P receptor. This could be tested by comparing PLF1 or MRP3 isolated from CHO cells with PLF1 or MRP3 isolated from 293 cells using the method described by Lee and Nathans on day 16 liver membrane preparations using the filters described in their procedure (4).

If the glycosylation pattern of PLF1 and MRP3 isolated from 293 cells leads to the absence of binding to the M6P receptor, it could also be responsible for the absence of binding
to the uterine membranes. This would suggest that the uterine receptor is sensitive to the state of glycosylation of the MRP/PLFs. Also, it is possible that a contaminant present in the conditioned medium of 293 cells co-purified with MRP3 and inhibited binding to the uterine and IGFII/M6P receptors.

Finally, the Appendix section has described the optimization of the rat lymphoma Nb2 cell prolactin receptor assay. This binding assay was designed to test if the removal of the carbohydrates from MRP/PLFs would stimulate the prolactin receptor in the NB2 cells to determine if the carbohydrates on MRP/PLFs are preventing the recognition of this prolactin-growth hormone family member to the receptor. This assay was to be used in parallel with the uterine membrane receptor assay to see if glycosylation could also regulate the activity of the MRP/PLF receptor, this would further identify and characterized the protein-receptor interactions with the uterine receptor.

The lipocalin 24p3/uterocalin was discovered similarly to MRP/PLF by adding growth factors to quiescent mouse fibroblasts 3T3 cells. However, unlike MRP/PLF, 24p3/uterocalin secretion is superinduced by the addition of the protein synthesis inhibitor cycloheximide. Like MRP/PLF, 24p3/uterocalin is expressed during gestation although MRP/PLF secretion is limited to the trophoblastic giant cells while 24p3/uterocalin is expressed in several tissues, both maternal and fetal.

Discovering the cell type(s) expressing 24p3/uterocalin in the liver, spleen and spine should help determine whether 24p3/uterocalin is expressed by the hematopoietic cells, like B-lymphocytes (13), neutrophils as shown in the human equivalent NGAL (14), other
hematopoietic cell types or possibly other cell types presently not known to express
24p3/uterocalin. 24p3/uterocalin could also be expressed in chondrocytes of the spine as
demonstrated in the rat and chicken homologues (15). The cell type(s) expressing the
24p3/uterocalin mRNA could be determined by in situ hybridization as preliminary
experiments suggest that the protein is not accumulating locally at levels detectable by
immunohistochemistry (Chapter 4) Also, because 24p3/uterocalin is a secreted protein, in situ
hybridization will be necessary to really establish the cell type(s) expressing it.

The results reported in Chapter 4 for the expression in the liver and spleen of juvenile
and adult mice were similar to the one obtained by Garay-Rojas et al. (16) with discrepancies
in the endpoint of the time course of expression. The different sex of the mice used in both
studies may have caused the observed differences in 24p3/uterocalin mRNA down-regulation
in the adults. Several proteins (17-20) are known to be differently regulated in the liver of
male and female. It is possible that 24p3/uterocalin is also differently regulated according to
the sex of the animal in the liver, spleen and spine. For both the liver and spleen, the
completion of expression profile during gestation and in the young mouse might also shed
light on the possible roles played by 24p3/uterocalin in these organs.

During late embryogenesis, the fetal liver is the site of hematopoiesis and at birth the
hematopoiesis is mainly relocated to the bone marrow of several bones. To further study a
possible role of 24p3/uterocalin for hematopoiesis in the liver, the co-culture of fetal liver
cells and pre-hepatic hematopoietic stem cells could be used to examine the effects of
24p3/uterocalin in these cell types. Takeuchi et al., using an immature hepatocyte and
hematopoietic stem cell co-culture, showed that the immature hepatocytes could support the
proliferation and maturation of hematopoietic stem cells in the liver. However, hepatocytes, as they mature in culture, can no longer support hematopoiesis. Thus the hematopoietic stem cell population will migrate to the bone marrow (21). The role of 24p3/uterocalin in the liver during hematopoiesis may be examined in this cell culture system by looking at expression levels of 24p3/uterocalin in the different cell types and observing whether the addition of purified 24p3/uterocalin to the liver and hematopoietic cells perturbs the ability of fetal hepatocytes to support hematopoiesis in vitro.

REFERENCES:


APPENDIX.

DEVELOPMENT OF A LACTOGENIC RECEPTOR BINDING ASSAY TO TEST DIFFERENT PROLACTIN-GROWTH HORMONE FAMILY MEMBERS

ABSTRACT:

MRP/PLFs are secreted glycoproteins of 34 kDa belonging to the prolactin-growth hormone family. MRP/PLFs are synthesized by the placenta, more precisely by the trophoblastic giant cells, during a short period of time ranging from day 10 to day 13 of gestation. A receptor for MRP/PLFs was found in vitro and maximum binding activity was observed on day 11 uterine membrane preparations. Maternal-fetal communications can be achieved, through the receptor for MRP/PLFs when the placenta (fetus) sends a signal (MRP/PLFs) to the uterus (mother). The nature of the signal appears to be stimulation of uterine cell proliferation as demonstrated by the increase in $^3$H-thymidine incorporation by day 11 uterine primary cell cultures. Reported here are data showing the modification of the Nb2 cell prolactin binding assay. The assay time was decreased from 72 h to 15 h using $^3$H-thymidine incorporation instead of cell counting to determine cell proliferation after stimulation by a lactogenic hormone. The Nb2 cell prolactin binding assay was used to show that MRP/PLFs did not act through the prolactin receptor. The data also showed that prolactin did not act through the MRP/PLF receptor, but prolactin inhibited the effect of MRP/PLF on a uterine primary cell system.
INTRODUCTION:

This appendix summarizes the research I did during May-July, 1994 in the Nilsen-Hamilton laboratory. This research provided the data which was used as the supporting thesis for the Master's of Science and Technology in "Biochemical and Biological Engineering" degree obtained in September 1994 from the Université Paris XII Val de Marne. For this appendix, I wrote an Introduction section different from the original thesis, revised the text for the Materials and Methods, and Results sections and rewrote the Discussion section with updated references.

Rat lymphoma Nb2 cells were derived from malignant lymphomas that arose in the lymph node of a Noble (Nb) male rat which had been treated with estrogen. Gout et al. established a suspension cell culture from a singular node, Nb2 (1). It was found that the Nb2 cells doubled every 15 hours in medium containing fetal calf serum, but when the media was switched to horse serum (HS) the cells stopped replicating. These stationary cells could resume growth in HS upon the addition of prolactin (PRL) (1, 2). Tanaka et al. used this property to develop a specific bioassay for binding ligands to the prolactin receptor based on the resumption of growth of the stationary cells by the addition of lactogenic hormones (2). The previous bioassays for the detection of prolactin receptor binding involved the pigeon crop sac stimulation assay (3), in vitro mammary gland cell cultures (4), or radioreceptor binding assay with $^{125}$I-labeled PRL on mammary gland membrane extracts (5). These assays were not very sensitive, technically difficult and had tedious methodology.

The Nb2 cell bioassay was used to test the role played by the glycosylation of MRP/PLFs in receptor recognition. Prolactin, placental lactogen and growth hormone do not bind to the
uterine receptor for MRP/PLFs (6). Conversely, MRP/PLFs do not have lactogenic activity, i.e. do not activate the prolactin receptor as demonstrated by the pigeon crop assay and the Nb2 cell bioassay (7). The high amino acid homology between all the prolactin-growth hormone family members suggests a similar three-dimensional protein structure. Prolactin, placental lactogen and growth hormone can all bind to the prolactin receptor (2). MRP/PLFs are heavily N-glycosylated compared to the hormones that can bind to the prolactin receptor. To investigate the role of glycosylation in receptor binding and activation, a chemical deglycosylation method was used to deglycosylate MRP/PLFs (7). This deglycosylated MRP/PLF was tested in both the uterine primary cell culture assay (MRP/PLF receptor binding), and the Nb2 cell bioassay (prolactin receptor binding).

In this appendix, the successful modification of the Nb2 cell bioassay provided a faster Nb2 cell bioassay decreasing the assay time from 72 hours to 15 hours by the use of $^3$H-thymidine incorporation by the cells resuming growth rather than single cell counting. The absence of MRP/PLF binding to the prolactin receptor was verified. Also shown is the preliminary data on the possible effect of prolactin on the uterine primary cell culture response to MRP/PLFs. The chemical deglycosylation method showed that it would require milligram amounts of MRP/PLF to yield sufficient deglycosylated protein for further testing.

MATERIALS AND METHODS:

*Animals and reagents*

The mice used for the uterine primary cell cultures were from the CF-1 strain. The animals were cared according to the “NIH guidelines for the care of laboratory animals” with
free access to food and water. The experimental protocols were approved by the Committee on Animal Care and Use of Iowa State University.

Nb2 cells were a gift from D. Prentice (Indiana State University, Terre Haute, IN).

MRP/PLFs were purified in the laboratory from conditioned medium of a BN/L cell line or a transfected (by bacculovirus) insect cell line. Mouse prolactin was a gift from A.F. Parlow (Harbor-UCLA Medical Center, Torrance, CA). Mouse placental lactogen I (PL-I) was a gift from F. Talamentes (University of California, Santa Barbara, CA). Ovine prolactin o-PRL from pituitary gland and porcine growth hormone (pGH) were purchased from Sigma. Rabbit anti-MRP/PLF antiserum was prepared in the laboratory using purified MRP/PLFs from conditioned medium of Swiss 3T3 cells. All the sera were purchased from Hy-Clone, except for the horse serum used for the stationary medium of Nb2 cells which was purchased from Gibco Invitrogen Corporation. Gibco was also the source of tissue culture supplies unless specified otherwise. The different HS tested for the stationary medium were from Hyclone (1 lot), Gibco Invitrogen Corporation (1 lot), Sigma (2 lots). Cells were counted using a hemocytometer or a Coulter counter®. Radioactivity uptake in tissue culture samples was determined using 5 ml of Scintiverse BD cocktail (Fisher) and counted in a Liquid Scintillation Analyzer (Packard 1900TR).

Coverslips used for cell growth were boiled in 1 % Calgon-metasilicate (8.8 g/l Calgon, 79 g/l sodium metasilicate), rinsed, boiled 10 min and rinsed in distilled water, then absolute alcohol. After drying, the coverslips were sterilized 4 h at 350 °C.
**Nb2 cell bioassay**

The cells were grown and prepared as previously described by Tanaka et al. (2). The Nb2 cells were grown as suspension cultures in 25cm² flasks (Costar) in growth medium (Fischer's medium supplemented with 10% horse serum, 10% calf serum, 10⁻⁴ M 2-mercaptoethanol, 20 units/ml penicillin/streptomycin). All incubations were done at 37 °C in 5% CO₂.

Nb2 cells were induced in the stationary phase by transferring them to a "stationary" medium (growth medium without calf serum) for 24 h to slow down their rate of replication. The cells were collected by centrifugation (10 min at 1000 x g), and resuspended in "stationary" medium at a concentration of 5 x 10⁵ cells/ml, and 1 ml was aliquoted in each well of a 24 well tissue culture plates (Costar).

Samples to be assayed for prolactin binding activity were incubated with the stationary cells for 9 hours followed by an additional 4 hours with 4 μCi/ml of methyl-³H-Thymidine (Amersham). Thymidine incorporation was stopped by centrifuging the cells for 10 min at 10,000 x g and the cell pellet was incubated at 0 °C with 10% triChloro-acetic acid (TCA) for 2 min. The mixture was vortexed and filtered on borosilicate microfiber filters (MFS). The filters were washed with 5 ml of 10% TCA followed by a wash with 95% ethanol, air dried, and the TCA precipitable counts were determined by scintillation counting.

**Primary uterine cell culture**

The method used in the laboratory was adapted by Nitza Rosenzweig from the method described by Ghosh et al. (8). Uteri from CF-1 mice at day 11 of gestation were collected aseptically in Ca²⁺, Mg²⁺ free Hank's balanced salt solution (HBSS). The uterine horns were
slit open to remove the embryos and placentae, cut into small pieces, and placed in a sterile
trypsin solution (0.1 % type III bovine trypsin (Sigma), 0.03 M EDTA in HBSS) for 20 to 24 h
at 4 °C. The trypsin solution was removed, and the uteri were incubated at 37 °C for 1 h.
Warm HBSS plus Ca^{2+}, Mg^{2+} was added and the tissue was vortexed for 5 min at high speed.
The dissociated tissue was allowed to settle at 1 x g for 5 minutes in the bottom of a 50 ml
centrifuge tube and the cell suspension was collected and centrifuged (10 min at 1000 x g).
The cell pellet was resuspended in 5 ml of HBSS plus Ca^{2+}, Mg^{2+} containing 0.3% BSA.
Clumps and sheets of epithelial cells were allowed to settle to the bottom of a 5 ml centrifuge
tube, at 37 °C, for 5 min. The supernatant containing the fibroblasts, endothelial, muscle and
stromal cells was carefully transferred and centrifuged 10 min at 1000 x g to collect the cells.

Freshly isolated cells were resuspended in growth medium, DMEM high glucose with
10 % heat inactivated fetal calf serum, 10 ng/ml porcine insulin (Sigma), and 20 units/ml
penicillin/streptomycin. The cells were plated at a density of 1.25 x 10^5 cells/well on coverslips
(1% Calgon-metasilicate treated) in 24 well plates and incubated at 37 °C in 10 % CO_2. After
24h, an equal volume of fresh medium was added and then half of the medium was changed
every two days. Typically, the cells were confluent after two weeks.

Half the medium was removed from confluent cell cultures and replaced with medium
containing the various reagents to be assayed. The cells were incubated for 30 h followed by
additional 4 hours with 8 μCi/ml of methyl-^3H-thymidine. The cells were washed in TBS
(140 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.68 mM CaCl_2, 0.5 mM
MgCl_2 pH 7.5) and fixed in 3.7 % formaldehyde, 0.11 M Na_2SO_4, 0.09 M NaCl overnight at
RT. The fixed cells on coverslips were incubated in ice cold 10 % TCA for 30 min, followed
by two washes with 95% ethanol and air dried. The amount of methyl-\(^{3}\)H-thymidine incorporated by the cells was then determined by scintillation counting.

**Chemical deglycosylation with TFMS**

MRP/PLF (approximately 20 \(\mu\)g) plus other glycoproteins (to have a total of 40 \(\mu\)g of protein) were dried under vacuum at room temperature. The dried proteins were treated with 3 \(\mu\)l of anhydrous Trifluoro methane sulfonic acid (TFMS) (Sigma) (9). The reaction mixture was incubated with occasional shaking at 0 °C for 1 h under nitrogen in a 1.5 ml tube. Subsequently, the reaction mixture was cooled at -20°C (in a dry ice-ethanol bath) and neutralized by gradually adding 60% pyridine (cooled at -20°C) to prevent overheating as the neutralization is exothermic.

**SDS polyacrylamide gel electrophoresis**

Samples to be separated by SDS PAGE were loaded on a 12% acrylamide gel and electrophoresis was completed at 12.5 mA for 6 h in 0.4 M Glycine, 50 mM Trizma, 1% SDS pH 8.35. The molecular weight markers were bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (33 kDa), Myoglobin (18 kDa), Cytochrome c (12.5 kDa). Proteins bands were detected by Coomassie blue followed by silver staining (10).

**Western Blot Analysis**

Samples containing MRP/PLFs were first resolved by SDS-PAGE. The proteins were transferred onto nitrocellulose membranes (Nitrobind 0.45 \(\mu\)m, Osmonics) in 25 mM Trizma,
190 mM Glycine and 20% Methanol pH 8.3, using a Hoefer TE50 apparatus at 360 V for 1 h at 4 °C. The membrane was stained with 0.2 % Ponceau S in 3 % trichloroacetic acid to ascertain uniform protein transfer to the membrane. After destaining with water, the membrane was incubated overnight at 4 °C in blocking solution (5 % wt/vol nonfat milk in 0.14 M NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 25 mM Tris, pH 7.5, 0.02 % wt/vol Na azide). The blocking solution was removed and replaced with fresh blocking solution containing a rabbit anti-MRP antiserum (recognizing PLF1, PLF2, MRP3, MRP4) at a final dilution of 1/200. After incubation at room temperature for 90 min, the membrane was washed three times (10 min each) in wash buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.5). The first wash contained 0.1 % Tween 20. The membrane was then incubated for 30 min at room temperature with goat anti-rabbit IgG conjugated to peroxidase (Pierce) diluted 1:5000 in wash buffer. The membrane was washed three times (10 min each) in wash buffer, with the first wash containing 0.1% Tween 20. The presence of peroxidase activity was detected by electro-chemiluminescence using ECL™, (Amersham Pharmacia) and exposing to x-ray film (Kodak).

RESULTS:

Characterization of the Nb2 cell culture

The initial growth curve of Nb2 cells obtained in the laboratory provided the growth characteristics of the particular clone obtained. The cell counts over the span of the growth curve were determined with a hemocytometer or a Coulter counter™. Figure 1 represents a typical growth curve for Nb2 cells.
The results from three different experiments gave an average cell doubling time of $15 \pm 1.5$ hours. It was noted that the cells stopped growing and began dying at a concentration of $2 \times 10^6$ cells/ml. However, when the cells were transferred to fresh medium close to the time they reached this critical cell number, the growth resumed.

The fetal calf serum dependency of Nb2 cells was tested by growing them in medium supplemented with different percentages of fetal calf serum and calf serum (Figure 2). There were no major differences in the doubling times of the cells grown on media containing different percentages of fetal calf serum and calf serum, as was reported previously by Gout et al. (1). It was established that Nb2 cells can be grown equally well in a medium supplemented with calf serum instead of fetal calf serum. In addition, the doubling times for this experiment were higher than the one previously determined, but this difference might be
explained by the fact that during this experiment the cells were incubated in an CO₂ atmosphere greater than 5%.

![Bar graph showing DNA Synthesis time for different percentages of calf serum.](image)

**Figure 2: Determination of the need for FCS by Nb2 cells.**
The doubling times of Nb2 cells grown on medium containing different percentage of calf serum (CS) and fetal calf serum (FCS), were determined in duplicate. 1: 10% FCS, 0% CS, 2: 8% FCS, 2% CS, 3: 6% FCS, 4% CS, 4: 4% FCS, 6% CS, 5: 2% FCS, 8% CS, 6: 0% FCS, 10% CS. The cells were counted with a Coulter Electronic Cell Counter™ (threshold 8μm to 15μm).

**Optimization of the modified Nb2 cell bioassay**
The first parameter optimized was the volume of 10% TCA needed to wash the filters and remove unspecific radioactivity. Four μCi/ml of ³H-Thymidine were added to growing Nb2 cells for 4 hours and the incorporation was determined using the protocol described in Materials and Methods with different repeats of 5 ml washes. Figure 3 shows that one wash with 5 ml of 10% TCA was enough to diminish the non-specific radioactivity bound to filters and have a percentage of error less than 20%. It was also determined that the centrifugation
step was necessary to remove the serum before doing the TCA precipitation as there were too many serum proteins present leading to the clogging of the filters.

![Graph](image)

**Figure 3: Determination of the volume of 10% TCA.**
Growing Nb2 cells were incubated with $^3$H-Thymidine for 4 hours. After centrifugation, they were incubated at 0°C with 10% TCA for 2 min, the radioactivity incorporated was measured by filtering the precipitate. The counts obtained on the filters after washing with the indicated volume of 10% TCA were the average of duplicates and the error bars represent the standard deviation.

The optimal concentration of the positive control ovine prolactin (oPRL) was determined. The cells were treated as described in the bioassay protocol with various concentrations of oPRL (Figure 4.) The saturation curve indicated that the 1 U/ml concentration was the minimal concentration of oPRL that gave the highest thymidine incorporation by Nb2 cells.
Figure 4: Determination of the optimal prolactin concentration.
Nb2 cells were treated with 0.01, 0.1, 1 and 6 U/ml of oPRL as described in Materials and Methods. Each point represents the mean of duplicate samples. The error bars represent the standard deviation. The background counts incorporated by non treated cells were 514 +/- 80.6.

The previous experiments were done with medium supplemented with HS that was known to make the cells stationary. Different lots of horse sera (see list of companies in Materials and Methods) were tested to identify a new lot of horse serum as efficient as the previous one. Figure 5 shows the disparity between different lots of horse serum tested. The best horse serum was determined as the one giving the lowest incorporation of $^3$H-Thymidine by the stationary cells and the highest response when treated with oPRL. The cell viability and ability to respond to prolactin showed that horse serum (HS2 from Gibco Invitrogen Corporation) was the best.
Figure 5: Screening of horse sera for the stationary medium. The Nb2 cells were resuspended in different stationary medium supplement with the different horse serum. Buffer (white) or 1 U/ml ovine prolactin (grey) was added to the cells which were incubated 20 hours followed by 4 μCi/ml of $^3$H-Thymidine addition for 4 hours. The incorporated radioactivity was then measured by TCA precipitation and filtration. The values expressed are means of duplicates.

All the previous prolactin experiments were done with an incubation time of twenty hours. Twenty hours was chosen considering the 15 hour doubling time of the cells and that cell division would resume with a slight delay. Determination of the optimal incubation time for $^3$H-thymidine incorporation by the Nb2 cells was the final experiment done to optimize the modified prolactin receptor Nb2 cell assay (Figure 6).

Maximal thymidine incorporation was clearly seen after nine hours of incubation with prolactin followed by a 4 hour pulse with 4 μCi/ml of $^3$H-thymidine.
Figure 6: Determination of the incubation time with prolactin for the highest thymidine incorporation. Stationary Nb2 cells were treated with or without 1 U/ml of 0-PRL. At each time point the cells were treated according to materials and methods. DNA synthesis is represented as fold-over control (cpm stimulated / cpm non-stimulated).

Effect of MRP/PLF and Prolactin on the uterine primary cell culture

From previous experiments, an increase in DNA synthesis was seen in cultures of uterine cells from day 11 of gestation uteri which were treated with MRP/PLFs (6). Figure 7 shows the effect of MRP/PLFs and mouse prolactin (mPRL) on uterine primary cell cultures.

Mouse prolactin added at two different concentrations (5 ng/ml and 1 μg/ml) did not affect the DNA synthesis of the primary uterine cells, whereas MRP/PLFs increased DNA synthesis at a concentration of 5 ng/ml. However, when 5 ng/ml or 1 μg/ml of mPRL are added at the same time as 5 ng/ml of MRP/PLF, DNA synthesis in the primary uterine cells is decreased in a concentration dependent manner.
Figure 7: Effect of MRP/PLF and prolactin on uterine primary cell cultures.
Uterine cells plated at 1.25x10^5 cells per well were grown for 2 weeks to confluency in 14 days and were incubated for 30 hours with 1: buffer, 2: MRP/PLF 5ng/ml, 3: mPRL 5ng/ml, 4: mPRL 1μg/ml, 5: MRP/PLF 5ng/ml + mPRL 5ng/ml, 6: MRP/PLF 5ng/ml + mPRL 1μg/ml. The results are expressed as fold-over control (cpm incorporated by the cells incubated with a molecule over cpm incorporated by the cells incubated with the buffer), the error bars indicates the standard deviations from duplicates.

Interaction of different molecules with the prolactin receptor

To verify whether MRP/PLF could act through the prolactin receptor to stimulate DNA synthesis, MRP/PLFs and the other members of the prolactin/growth hormone family were tested in the Nb2 bioassay (Figure 8).

This experiment showed that mouse prolactin, mouse placental lactogen I and porcine growth hormone, stimulated growth of the Nb2 cells, whereas MRP/PLFs did not. Also, MRP/PLFs (5 ng/ml and 0.1 μg/ml) were added with mPRL (5 ng/ml) to see if MRP/PLF could modulate binding of mPRL to the prolactin receptor. Figure 8 shows that MRP/PLF does not affect the effect of prolactin on the growth of Nb2.
Figure 8: Effect of various members of the PRL-GH family on the Nb2 cells.
Stationary Nb2 cells were incubated 9 hours with 1: buffer, 2: oPRL 1U/ml, 3: mPL-1 5ng/ml, 4: pGH 1 U/ml, 5: mPRL 5ng/ml, 6: mPRL 5ng/ml + MRP/PLF 5ng/ml, 7: mPRL 5ng/ml + MRP/PLF 100ng/ml, 8: MRP/PLF 5ng/ml, 9: MRP/PLF 100ng/ml. DNA synthesis is expressed as fold-over control (cpm with treatment over cpm with no treatment) and the error bars indicate the standard deviation from duplicates.

Chemical deglycosylation of MRP/PLFs with TFMS

Twenty micrograms of MRP/PLF was added to a mixture containing 5 μg of each of the following proteins: fibronectin, BSA, ovalbumin and cytochrome c. The different proteins were added to MRP/PLFs to have a total amount of protein equal to 40 μg (minimum amount of protein recommended for TFMS deglycosylation. The protein mixture containing MRP/PLF was treated as described in Materials and Methods. Figure 9 shows that there is no detectable accumulation of deglycosylated MRP/PLFs in the reaction mixture. However, TFMS treatment leads to the disappearance of MRP/PLFs suggesting that it was degraded during the chemical deglycosylation.
DISCUSSION:

The Nb2 cell bioassay was successfully modified from the previously described assay used to detect binding to the prolactin receptor (1, 2). The modifications developed here decreased the assay time from 72 h to approximately 15 h.

Prolactin inhibited DNA synthesis stimulated by MRP/PLFs, in a concentration dependent manner, on uterine primary cell cultures. However prolactin does not bind to the
MRP/PLF receptor (6). Therefore Prolactin could act through another receptor on the cells that are responsive to MRP/PLFs thus acting on a pathway which would inhibit the action of MRP/PLFs. Alternatively, prolactin could induce a different cell type also present in primary uterine cell cultures to synthesize molecules that will decrease the DNA synthesis of the MRP/PLFs stimulated cells. Corroborating evidence could show a role for MRP/PLF regulation by prolactin or a lactogenic hormone (most likely placental lactogen I and II (11)) which are secreted during mid-gestation by the placenta.

The absence of stimulation by MRP/PLFs of the Nb2 cells confirmed the finding that MRP/PLFs can not act through the prolactin receptor (7). Furthermore, MRP/PLFs did not decrease the activity of mPRL in the Nb2 cell. The high degree of homology between the primary protein structures of MRP/PLFs, prolactin and growth hormone suggest that the tertiary structures would be similar and MRP/PLFs could bind to the lactogenic receptor like the classical members of the prolactin/growth hormone family (12). One hypothesis is that the heavy glycosylation of MRP/PLFs would prevent the binding to the lactogenic receptor. The difference in glycosylation of prolactin could also explain its inability to bind to the MRP/PLF receptor observed in previous experiments (6).

To identify the roles of glycosylation, deglycosylated MRP/PLF would be required for the Nb2 cell bioassay. The chemical method was the most economical and the fastest to perform compared to the use of endoglycosidases. Also as time was a factor, site-directed mutagenesis or bacterial expression (because a new purification process would have to be developed) was not considered as the first option. Unfortunately, the TFMS method degraded MRP/PLFs significantly and the incubation time used might not have been long enough to
detect the accumulation of deglycosylate MRP/PLFs. At the time, not enough MRP/PLFs was available to optimize the chemical deglycosylation to obtain deglycosylated MRP/PLFs.

In summary, the batch of MRP/PLFs isolated from BN/L cells used in these experiments stimulated uterine cell proliferation whereas mPRL did not. Interestingly, mPRL inhibited the effect of MRP/PLFs on uterine primary cell cultures. Finally, MRP/PLFs isolated from BN/L cells did not activate the prolactin receptor as was reported before for recombinant PLF1 isolated from CHO cells (7).

REFERENCES:


