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THE HUMAN FETAL MEMBRANES, DECIDUA AND PLACENTA
AS PARACRINE SYSTEM

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE
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IN
BIOMEDICAL SCIENCES
(ANATOMY AND REPRODUCTIVE BIOLOGY-
CELL, MOLECULAR AND NEUROSCIENCE)

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ABSTRACT

The full characterization of a hormone requires knowledge of its chemical structure and control of its synthesis/cells of origin, its mode of transmission, the localization and structure of its receptor and binding proteins, the activation of its second messengers and its modulation of the transcription of specific genes in specific tissues-its biological effect.

The work presented here offers significant insights into two peptide hormones of known structure, prolactin (PRL) and relaxin (RLX), shown by others to be products of the maternal decidua and to transverse the juxtaposed fetal membranes into the amniotic fluid rather than into the systemic circulation.

A study of the receptor for PRL by the techniques of Northern analysis, in situ hybridization and immunocytochemistry has shown that the receptor is widely distributed in the maternal-fetal compartment and in the placenta and points to major autocrine and paracrine targets for decidual PRL.

A new area of investigation has been opened up by applying the technique of Western blot analysis to separate PRL receptor (PRL-R), related proteins produced at the different sites of translation since, in addition to the classical 95-85 kDa membrane-bound PRL-R, additional species in tissue lysates and in the media of tissue explants have been identified. Further studies may give insights into the processing of the receptor intracellularly and/or lead to the novel demonstration that these proteins are involved in the transport mechanisms necessary, in addition to the PRL-Rs, in the amnion, chorion and

decidua to direct the decidua PRL to the fetal compartment rather than to the maternal myometrium and systemic circulation.

The presence of a receptor for the hormone RLX in the human chorio-decidua has been confirmed after almost a decade since it was first demonstrated, using chemically synthesized human RLXs H1 and H2 and a new method of labeling which preserves receptor binding activity. The binding of $\gamma^{32}\text{P}$ -labeled RLX H2 to chorio-decidua enriched membrane particulate fractions has been optimized. The specificity of the binding of RLX to these particulate fractions and other characteristics of the binding have been completed. Evidence is presented for a single RLX-R target for both of the human RLXs produced in the decidua. The binding of human RLX H1, albeit with a 5 fold lower affinity than RLX H2 provides inferential evidence that the H1 RLX gene product is of biological significance.

Hence, the results indicate that like PRL, the human RLXs H1 and H2, elaborated by the human decidua, have autocrine/paracrine actions within the decidua and the fetal membranes and should stimulate further attempts to isolate and characterize the receptors for the RLXs and to study their distribution within the maternal-fetal compartment.

CO-existence of PRL and RLX and their receptors in the maternal-fetal compartment have been linked by the observation here that PRL may complement the role of RLX established by others on the release of specific metalloproteinases targeting the collagen of the extracellular matrix.

The modulation of the action of a hormone by changes in the concentration of its receptors, rather than in the tissue concentration of the hormone, has been shown. An increase in the transcription of the PRL-R gene has been demonstrated after normal spontaneous labor and delivery. If a similar increase in the concentration of RLX-R was established, a cascade mechanism for the initiation of the massive collagenolysis associated with and required after the expulsion of the fetus may be established.

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LIST OF ABBREVIATIONS

ABC	avidin-biotin immunoperoxidase complex
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
C-peptide	connecting peptide
cpm	counts per minute
CRH	corticotropin releasing hormone
DAB	diaminobenzidine
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
ER	endoplasmic reticulum
GF	growth factor
hCG	human chorionic gonadotropin
IgG	immunoglobulin G
kb	kilobases
M	moles
Mab	monoclonal antibody
mmol	millimoles
mRNA	messenger ribonucleic acid
ng	nanogram
NIH	National Institute of Health

³² P	radioactive phosphorus
PBS	phosphate buffered saline
poly(A) ⁺	polyadenylic acid
RIA	radioimmunoassay
³⁵ S	radioactive sulfur
SDS	sodium dodecyl sulfate
SSC	sodium saline citrate
SSPE	sodium chloride, sodium phosphate and EDTA
TdT	terminal deoxynucleotidyl transferase
TRH	thyrotropin releasing hormone
tRNA	transfer ribonucleic acid
ug	microgram
ul	microliter

CHAPTER I INTRODUCTION

A. The fetal membranes, decidua and placenta

The endometrium is unusual in its ability to undergo cyclic morphological and biochemical changes during a woman's reproductive years. This tissue is composed of a diverse population of cells with distinctive programs of proliferation and differentiation. Endometrial stromal cells undergo terminal differentiation into decidual cells (decidualization) during the luteal phase of the menstrual cycle. The stromal cells that surround blood vessels are the first to undergo decidualization, this process spreads throughout the endometrium when conception occurs. The decidual cells proliferate and become the main component of the pregnant endometrium, this gestational endometrium is known as the decidua. In addition to the epithelial and stromal cells, the decidua is composed of 47% bone marrow derived cells including, macrophages, T-cells and large granular lymphocytes (LGL), (Starkey, 1988). The function of these bone marrow-derived decidual cells are at present unknown, although they are thought to be involved in: (1) the regulation of the maternal immune response to the fetal allograft, (2) the presentation of fetal antigens to the maternal immune system in order to invoke a protective response (3) serving to limit trophoblast invasion, (4) the remodeling of the extracellular matrix (ECM) and (5) the production of hormones, cytokines and prostaglandins (Kadanoff, 1993; Mizuno, 1994; Kliman, 1993) . This decidual tissue becomes enlarged and metabolically active exhibiting significant changes in cellular composition as pregnancy continues.

The maternally derived decidua, depending on anatomic location, is composed of three major parts shown in Fig. 1: (1) the decidua basalis, which underlies the site of implantation and forms the maternal component of the placenta; (2) decidua capsularis, which overlies the gestational sac (this portion disappears in the later stages of pregnancy); (3) decidua parietalis or decidua vera, which lines the remainder of the uterine cavity and becomes adhered to the chorion (Fig. 1).

The fetal membranes begin to differentiate by day 7 post conception, as the trophoblast contacts the endometrium, it proliferates and differentiates into two layers, the inner cytotrophoblast layer and the outer syncytiotrophoblast layer (syncytium). By day 8 the ectoderm derived amniotic cavity is formed in the inner mass of the invading trophoblast. As pregnancy progresses these layers further differentiate to form the chorionic villi of the placenta, chorion laeve and the amnion. The placenta develops at the site where the embryo attaches to the uterine wall, the syncytiotrophoblast of the placenta is highly invasive; as a result, there is considerable necrosis of the decidua basalis and deposition of fibrinoid material that separates the fetal and maternal parts of the placenta (the Nitabuch's layer). The growing chorionic sac associating with the decidua capsularis and compresses causing the degeneration of the chorionic villi thereby forming a relatively avascular smooth bare area. The amniotic sac enlarges somewhat faster than the chorionic sac causing the fusion of their walls. The amnion is a strong, thin, transparent membrane that envelops the developing fetus, it is in contact with and produces amniotic fluid (Eastman and Hellman, 1961; Sanders, 1975).

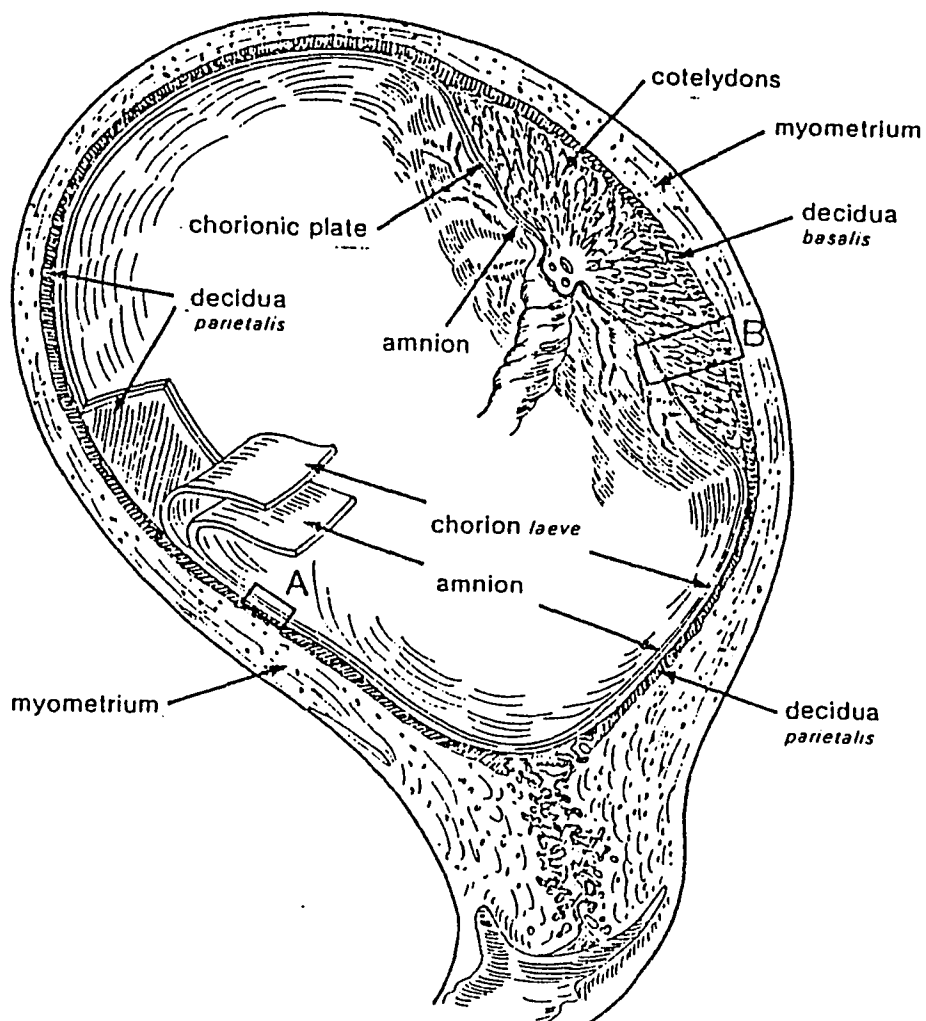


Figure 1. Full term placenta in utero.

A and B: sections for immunocytochemistry.

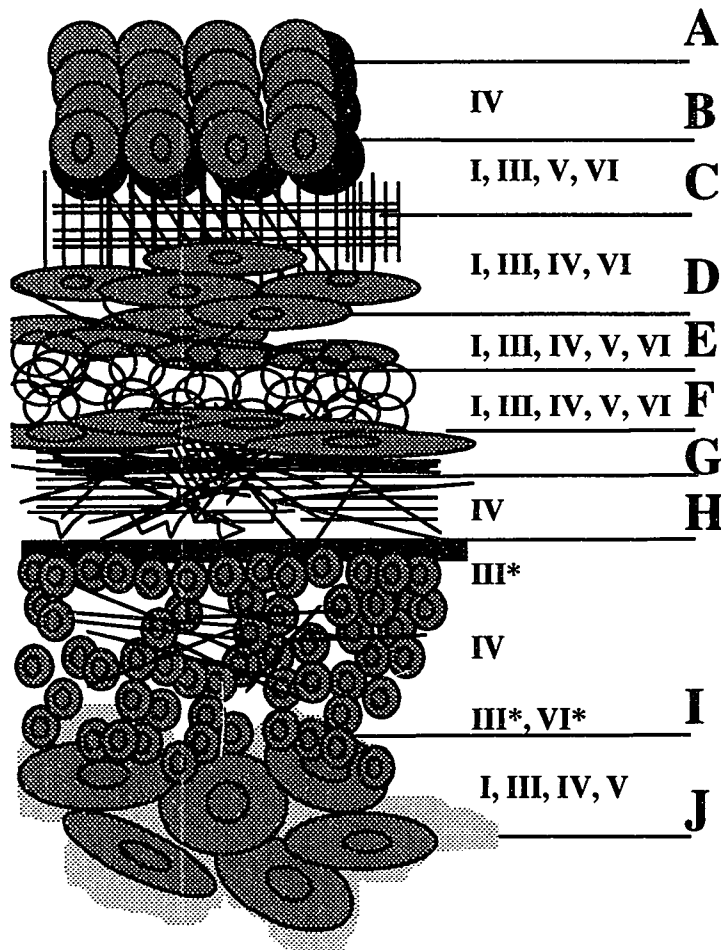
from: Williams Obstetrics, 16th edition, 1980.

1. Structural aspects of term fetal membranes, decidua and placenta

The most important functional property of the fetal membranes, decidua and placenta is conditional on its structural and subsequent mechanical integrity, that is its ability to maintain the fluid environment in utero for the developing fetus. The fetus's survival is incumbent upon the cellular response of the membranes (fetal and maternal) to their local environment of connective tissue and extracellular matrices (ECM). This network of connective tissue, pseudo-basement membrane and basement membrane along with its ECM degrading enzymes and their inhibitors are most important in the maintenance of the non-hostile utero environment. This ECM architectural framework for the amnion, chorion and decidua is shown in detail in Fig. 2.

a. The amnion

The human amnion at term is comprised of 5 layers; (1) the epithelium, (2) the basement membrane, (3) the compact layer, (4) the fibroblast layer and (5) the spongy layer and is 0.2-0.5mm in thickness (Fig. 2) (Bourne, 1960). The amniotic epithelium is columnar in appearance covered by numerous microvilli containing irregular apical borders, intercellular channels and nuclei located near the apex of the cell (Van Herendael, 1978; Bourne, 1960). The basement membrane is a narrow band of connective tissue consisting mainly of type IV collagen running along the base of the amniotic epithelial cells (Malak, et al., 1993). Short, blunt processes from the bases of the epithelial cells interdigitate with laminar processes that arise from the laminar rara and laminar densa of this basement membrane (Aplin, et al., 1985). The compact layer of the amnion is



COLLAGEN TYPES: I, III, IV, V, VI
 *denotes low level

Amnion: A, amniotic epithelium
 B, basement membrane
 C, compact layer
 D, fibroblast layer
 E, spongy layer

Chorion: F, cellular layer
 G, reticular layer
 H, pseudo basement membrane
 I, trophoblast layer

Decidua: J, compact zone

Figure 2. Schematic diagram of fetal membranes and decidua, showing distribution of collagens

acellular and consists of a dense network of types I, III, V and VI collagen fibers (Malak, et al., 1993). This layer appears to be the strongest of the amniotic layers, containing fibrils of both 25 and 50nm in diameter (Aplin, et al., 1985). The fibroblast layer is predominately composed of types I, III, V and VI collagen. It is the thickest layer of the amnion, containing embedded fibroblast cells which can be phagocytically active (Bourne, 1960 ; Malak, et al., 1993). The spongy layer permits the amnion to slide upon the underlying chorion, this layer is comprised of types I, III, IV, V and VI collagens that form a loose, wavy network (Bourne, 1960; Malak, et al., 1993).

b. The chorion

The human chorion at term consists of four layers; (1) the cellular layer, (2) the reticular layer, (3) the pseudo-basement membrane and (4) the trophoblast layer (Fig. 2). The cellular layer is a thin, imperfect network of fibroblast cells, which is frequently absent in term chorionic membranes (Bourne, 1960). The reticular layer consists of types I, III, IV, V and VI collagens (Dieron and Bryant-Greenwood, 1991; Malak, et al., 1993). This is the thickest layer of the chorion, containing a reticular network of parallel fibers (Bourne, 1960). The pseudo-basement membrane contains type IV collagen, is firmly adherent to the above reticular layer and sends anchoring fibers down into trophoblast layer (Bourne, 1960 ; Malak, et al., 1993). The trophoblast layer consists predominately of type IV collagen, it is the deepest layer of the chorion, containing two to ten layers of cytotrophoblast cells. The upper layer of the cytotrophoblast cells adherent to the pseudo basement membrane do not contain type III and little if any types V and VI collagens,

however, the cytotrophoblast layer adherent to the decidua parietalis contains type V and small but detectable amounts of type III and VI collagens (Malak, et al., 1993).

c. The decidua parietalis: structure and components

The decidua parietalis is composed of three layers; (1) the compact zone that is in direct contact with the cytotrophoblast of the chorionic layer, (2) the intermediate layer or spongy zone, this layer contains tortuous glands and numerous small blood vessels and (3) the basal zone which lies along the maternal myometrium (Fig. 2). The functional zone of the decidua consists of the compact zone and the spongy zone, these layers are sluffed off during menstruation and shed following parturition, hence its name. The remaining basal zone gives rise to the new endometrium of the menstrual cycle and subsequent decidua (Eastman and Hellman, 1961).

The decidual cells are plump, epithelioid, eosinophilic and polygonal, arranged in a cobblestone configuration with distinct pericellular borders (Wewer, et al., 1985; Dieron and Bryant-Greenwood, 1991). The ultrastructural cytoplasm of these cells is characteristic of a hypersecretory state with an abundant amount of rough ER, large mitochondria and Golgi complexes containing numerous stacked saccules (Thrasher and Richart, 1972).

A basement membrane consisting of a network of types I, III, IV and V collagens, proteoglycans and other protein components cross-linked to form a pericellular matrix surrounding the decidual cells (Damjanov, 1985; Dieron and Bryant-Greenwood, 1991; Malak, et al., 1993) . A general interaction between cells and their surrounding ECM has

been well established in a number of other systems (Streuli, et al., 1993; Howlett and Bissell, 1993; Boudreau, et al., 1995). Matrix components, like fibronectin and laminin, which bind to specific receptors (integrins) on the basal plasma membrane of epithelial cells, are important in the establishment of the polarity of the cells in addition to other cell matrix signaling.

Decidua parietalis examined at term is composed of approximately 50% of the cell population were of bone marrow origin, comprising 18% macrophages, 3% LGL cells, 8% T cells and approximately 18% granulocytes (Vince, et al., 1990). It has been suggested that early decidual macrophages have a capacity for allo-antigen presentation and higher suppressive activity, as compared to their peripheral counterparts, two important roles in implantation and the maintenance of pregnancy. The $\gamma\delta$ T cells as opposed to their $\alpha\beta$ T cell counterparts appear in mid pregnancy and increase in number until delivery. These cells are believed to be important in reducing the number of fetal white blood cells passing through to the maternal circulation (Starkey, et al., 1988; Vargas, et al., 1993; Ditzian-Kadanoff, et al., 1993). Unfortunately, the characterization of the endometrial lymphoid system is relatively rudimentary and its exact function remains to be defined.

d. The decidua basalis: structure and components

The decidua basalis is anchored to the placenta at the Nitabuch's layer by junctional trophoblasts (Kilman, 1993). This anchoring allows the invasive trophoblast to migrate into the decidua basalis, thus providing direct contact with maternal arteries and

veins. In addition to the invading trophoblast cells and decidualized stroma cells, the decidua basalis contains bone marrow derived cells. There is conflicting evidence as to the type of leukocytes present at term, further studies are necessary to clarify these cell populations and their respective roles in the course of gestation and subsequent delivery (Vince *et al.*, 1990; Kilman, 1993; Vargas, *et al.*, 1993).

2. Dynamics of the fetal membranes, decidua and placenta

Thus the maternal decidua parietalis and basalis lies at the interface between the chorion and amnion of fetal origin and the adjacent maternal myometrium in the perfect location for the bi-directional passage of autocrine/paracrine signals between the fetus and the mother. Similarly, the developing fetus and its placenta forms another interdependent partnership in regulating endocrine-metabolic processes during the course of pregnancy. This functional relationship, known as an “organ communication system”, is a unique endocrine/paracrine system that produces a large number of bioactive agents and is also important in the initiation of parturition (Casey and MacDonald, 1991). These bioactive agents include proteolytic enzymes, peptides, neuropeptides, steroids, growth factors, prostaglandins and their receptors.

The bioactive agents produced are also responsible for the production, regulation and remodeling of the extracellular matrix during the course of pregnancy. There is a dynamic balance between synthesis and degradation of the different ECM components. Matrix metalloproteinases (MMPs) are one major group of proteolytic enzymes that specifically degrade ECM components (Emonard and Grimaud, 1990). Matrix degrading

proteinases have been shown to increase during dilatation of the human uterine cervix, there is strong evidence that this “ripening” of the cervix involves the partial breakdown of collagen fibers (Uldbjerg *et al.*, 1983). The tightly controlled connective tissue remodeling which takes place in the human fetal membranes has been proposed to be important for their accommodation to the growth of the intrauterine contents (Bryant-Greenwood, 1991). This remodeling is probably controlled by the locally acting decidual hormones like relaxin and prolactin.

B. Decidual hormones: PRL and RLX

1. Human Prolactin as an autocrine/paracrine hormone in the fetal membranes

decidua and placenta

a. Prolactin Gene

The PRL gene appears to have been derived from a common somatomammotrophic [growth hormone (GH), PRL, placental lactogen (PL)] precursor gene (Nial, *et al.*, 1971; Miller and Eberhardt, 1983) Fig. 3. With the recent reanalyses together with the additional data on peptide hormones belonging to this hormone family, it has been verified that PRL indeed belongs to the GH, PRL, PL family (Miller and Eberhardt, 1983). Prolactin is the product of a single gene located on chromosome 6, alternative splicing of its primary transcript leads to two very similar but distinct PRLs expressed in the decidual and pituitary (Owerbach, *et al.*, 1981).

Decidual and pituitary PRL proteins have identical chemical, biological and immunological properties, however, decidual PRL mRNA has an additional 150 bases

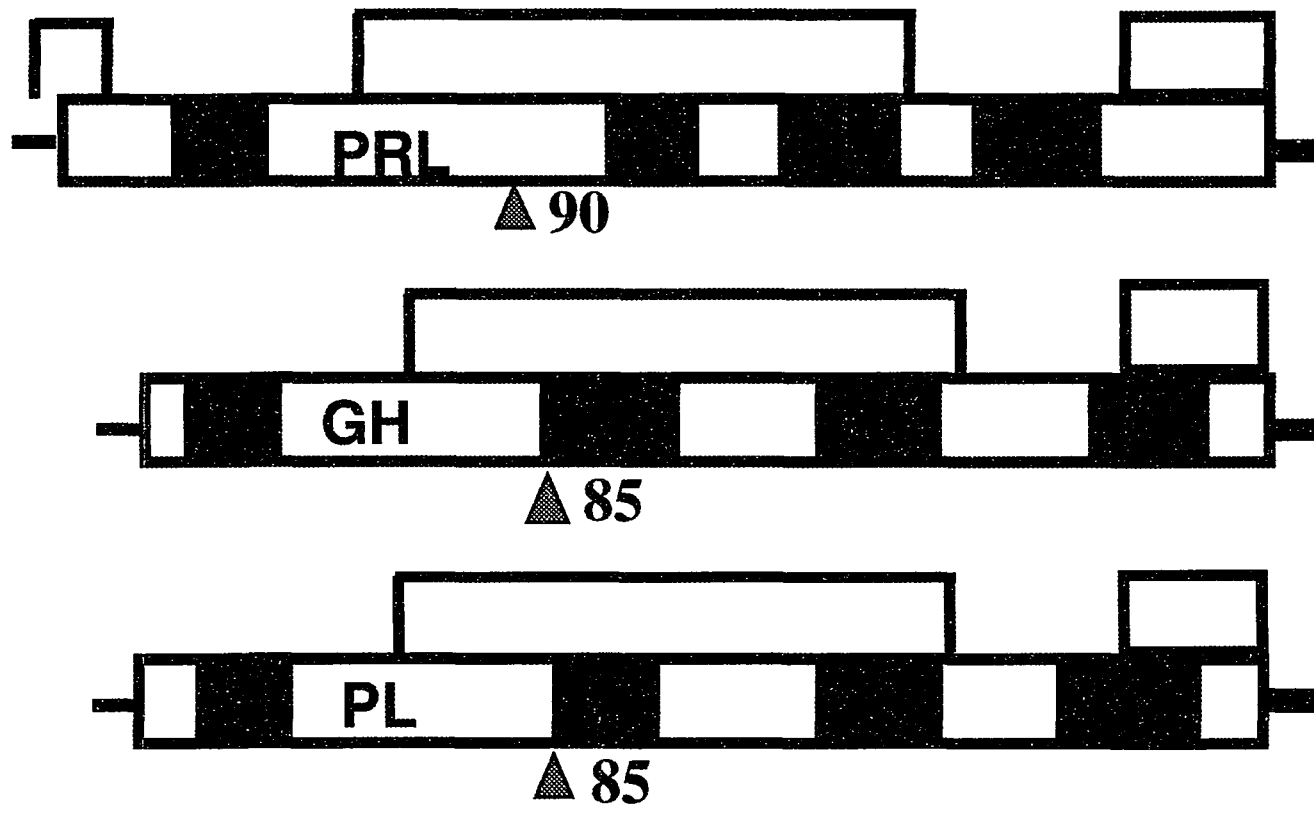


Figure 3. Structural similarities in the GH, PRL and PL super family. The black boxes are areas of high homology and the lines above each bar represent disulfide bridges (from Niall, HD *et al.*, 1971). Abbreviations: PRL: prolactin, GH: growth hormone, PL: placental lactogen; diagnostic tryptophan residues are numbered, triangle marks their location

residing in the 5' untranslated region (Golander et al., 1978; Gellerson, et al., 1989). This difference allows the differential transcriptional regulation of the PRL gene in these two tissues. Thus, thyrotrophin releasing hormone (TRH) stimulates and dopamine inhibits the release of pituitary PRL, but neither dopamine nor TRH affects the synthesis or release of decidual PRL (Bigazzi et al., 1979). The storage of PRL is also different in the two tissues, pituitary PRL is stored in secretory granules which, release the pituitary hormone from a readily releasable pool and newly synthesized PRL refills this as well as the storage pool upon exposure to secretagogues (Stachura, et al., 1989). Decidual PRL is rapidly released after synthesis and is not stored in dense granules but rather localized to the post microsomal supernatant (Handwerger, et al., 1984).

b. PRL peptide

Human prolactin (PRL) occurs in multiple glycosylated forms, '*little*' PRL (MW ~23,000), corresponding to the non-glycosylated monomeric hormone termed G-PRL¹ and a second G-PRL² (MW~25,000), corresponding to glycosylated monomeric hormone differing only in the carbohydrate units, '*big*' PRL (MW ~50,000), consisting of a mixture of dimeric and trimeric forms of glycosylated PRL, and *big-big* PRL (MW ~100,000), possibly representing G-PRL coupled covalently with immunoglobulin (Heffner et al., 1987). Glycosylated forms of PRL in serum are less immunoreactive than '*little*' PRL and are the predominant forms present in most human plasma (Markoff and Lee, 1987). The isoforms of PRL exhibit a shift from G-PRL¹⁻² to the more bioactive *little* PRL during the course of pregnancy in both serum and amniotic fluid (Markoff et al., 1988). Thus, G-PRL¹⁻² is the predominant form during the first trimester of pregnancy and *little* PRL

represents the major component of immunoreactive PRL at term (Markoff et al., 1988; Larrea et al., 1989). Therefore, in response to the changing hormonal milieu of pregnancy, the isoforms of PRL in serum shift in favor of the more biologically active form. Decidual explants of early pregnancy secrete a greater proportion of G-PRL¹⁻² as compared to term decidua, this same gestational pattern for G-PRL¹⁻² is present in amniotic fluid (Lee and Markoff, 1986; Markoff et al., 1988).

1. Serum and amniotic fluid levels in pregnancy

Maternal serum PRL begins to rise in the first trimester of pregnancy and increases in an approximately linear pattern to term, reaching ten fold the serum concentration found in cyclic women (Tyson, et al., 1972; Rigg and Yen, 1977; Jaffe, et al., 1983). In contrast, amniotic fluid PRL peaks in the second trimester of pregnancy (10-100 fold greater than that of maternal serum), when both the maternal and fetal PRL levels are relatively low (Schenker, et al., 1975) Fig 4. Several attempts to demonstrate a correlation between the human fetal or maternal serum PRL concentrations and amniotic fluid concentrations have been unsuccessful (Biswas, 1976; McNeilly et al., 1977 and Andersen and Weber, 1985). PRL was shown to be of unidirectional flow, from the decidua to the amniotic cavity providing the fetal membrane and adhering decidua are intact (Tyson et al., 1982) thus, it appears that decidual PRL does not contribute to the serum PRL pool.

The principal source of amniotic fluid PRL was initially thought to be either the maternal or fetal pituitary, however several lines of evidence pointed to an extrapituitary

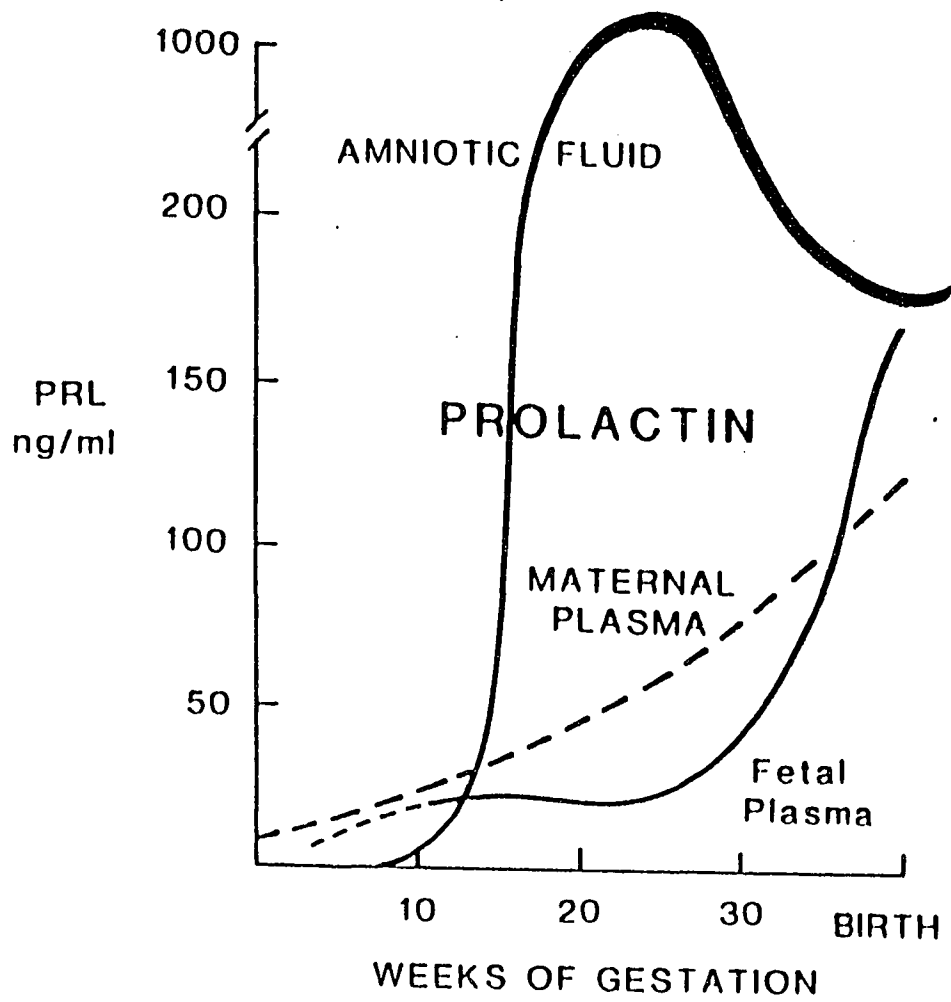


Figure 4. Prolactin levels during pregnancy

adopted from Schenker JG et al. Am J Obstet Gynecol, 1975

source for this PRL. No proteins as large as 20 kDa are known to cross from maternal serum through the placenta into the amniotic fluid at such high concentrations.

Bromocriptin administration to pregnant women lowered maternal serum PRL (pituitary PRL) levels but had no effect on amniotic fluid PRL levels (decidual PRL) (Bigazzi, *et al.*, 1976). Furthermore, PRL levels in the amniotic fluid of rhesus monkeys remained constant after either maternal hypophysectomy (removal of the maternal pituitary source for PRL) or fetal death in utero, (removal of the fetal source for PRL) (Josimovich, *et al.*, 1977). In addition, the media of cultured chorio-decidua explants showed a 35-fold increase in PRL over a 48 hour period, demonstrating unequivocally this tissue as a source of PRL (Healy, *et al.*, 1977), and its source in amniotic fluid (McCoshen and Tyson, 1985).

2. Physiological roles

The classical action of pituitary PRL is the regulation of milk proteins (casein and α -lactalbumin) and fat synthesis during lactation. During pregnancy the increasing levels of PRL, in addition to cortisol, PL, estrogens and progesterone combine to stimulate the development of secretory apparatus of the breast (Shiu and Friesen, 1980). The role(s) of decidual PRL during the course of pregnancy are less defined. Decidual PRL has been suggested to have a role in a number of intrauterine functions: 1. water and salt balance (Pullano, *et al.*, 1989); 2. the production of lecithin, (an active component of surfactant), indicating a possible role in fetal lung development (Hamosh and Hamosh 1977); 3. fetal thymic hormone production during the period in which fetal pituitary PRL is relatively low (Dardenne, *et al.*, 1989); 4. as an autocrine growth factor for mononuclear cells which

may promote changes in local cytokine production (Clevenger *et al.*, 1990; Sabharwal, *et al.*, 1992); 5. the production of prostaglandins, leading to possible stimulation of the myometrium (Gellersen, *et al.*, 1991).

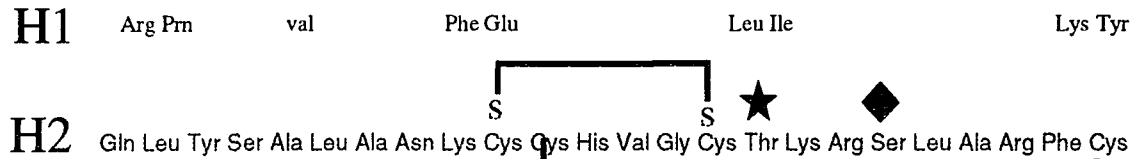
2. Human relaxin as an autocrine/paracrine hormone in the fetal membranes,

decidua and placenta

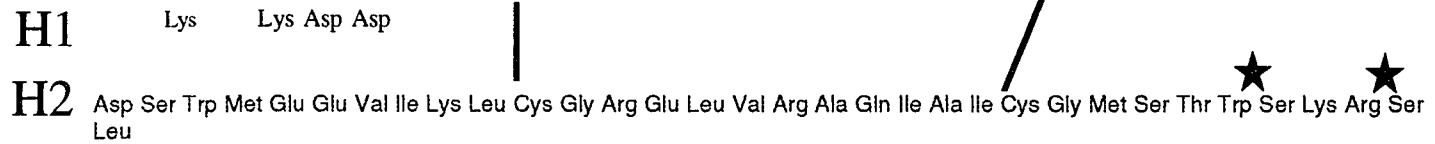
a. The human relaxin genes

The human genome has two nonallelic genes for RLX, termed H1 and H2 (Hudson *et al.* '83, '84) both found on chromosome 9 in the area containing the interferon gene cluster (Crawford *et al.*, 1984). The human relaxin gene was first identified from a genomic library by Hudson *et al.* in 1983, and was termed the RLX H1. In 1984 Hudson *et al.* identified a second form of human relaxin gene from a corpus luteum cDNA library which they termed human relaxin H2. These hormones share 92% homology at the nucleotide level and have very similar primary structures with 77% amino acid sequence identity (Hudson *et al.*, 1984) Fig. 5. The H1 and H2 RLX genes are expressed in the decidua, placenta and prostate, however the corpus luteum of pregnancy and of the menstrual cycle only expresses H2 RLX gene (Hansell, *et al.*, 1991). The absence of immunoreactive relaxin in the serum of women after ovum donation in the absence of ovaries suggests that the source of systemic RLX in the female is the corpus luteum (Emmi, *et al.*, 1991; Johnson *et al.*, 1991).

A Chain



B Chain



- ★ possible Serine phosphorylation sites for both H1 and H2 Relaxin
- ◆ possible Threonine phosphorylation site for H2 Relaxin

Figure 5. Amino acid sequence comparisons of human relaxins with possible phosphorylation sites denoted (Bryant-Greenwood and Schwabe, 1994)

b. The human relaxin peptide

An observation that serum from pregnant guinea pigs or rabbits caused the relaxation of the pubic ligament when administered to virgin guinea pigs shortly after estrus led to the suggestion of an hormonal moiety produced in pregnancy with this biological effect (Hisaw, 1926). By 1930 Fevold and Hisaw had obtained a crude preparation from porcine corpora lutea and the hormone was given the name RLX . RLX is hydrophobic in nature and the exceptional ability of its B-chain to adsorb to most surfaces made it very difficult to purify. However, in 1974 Sherwood and O'Byrne developed a procedure for isolating RLX in high yields using ovaries from pregnant pigs as the starting material.

The general structure of RLX is very similar to that of insulin and it is therefore classified in the insulin/insulin like growth factor (IGF) superfamily of hormones (Kemp and Niall, 1984) Fig. 6. The fundamental and general features of RLX, insulin, IGF and Leydig I-L a member of this family. The finer details of their structure show: (1) they are composed of an A and B chain (2) the location of the cysteine residues is conserved causing the disulfide bridges to have the same positions, (3) the overall 3-dimensional folding of RLX therefore resembles that of insulin and (4) the connecting peptide (C-chain) is cleaved out to form the mature hormone at dibasic residues located at the B/C chain junction and at the A/C chain junction after the formation of the disulfide bridges, the C-peptide ranges from 23 amino acids (A.A) to 311 A.A. in length for IGF and RLX respectively (Marriott, et al., 1992). In spite of this structural similarity, RLX and insulins' primary amino acid sequences differ by approximately 70% (Healy, et al., 1982)

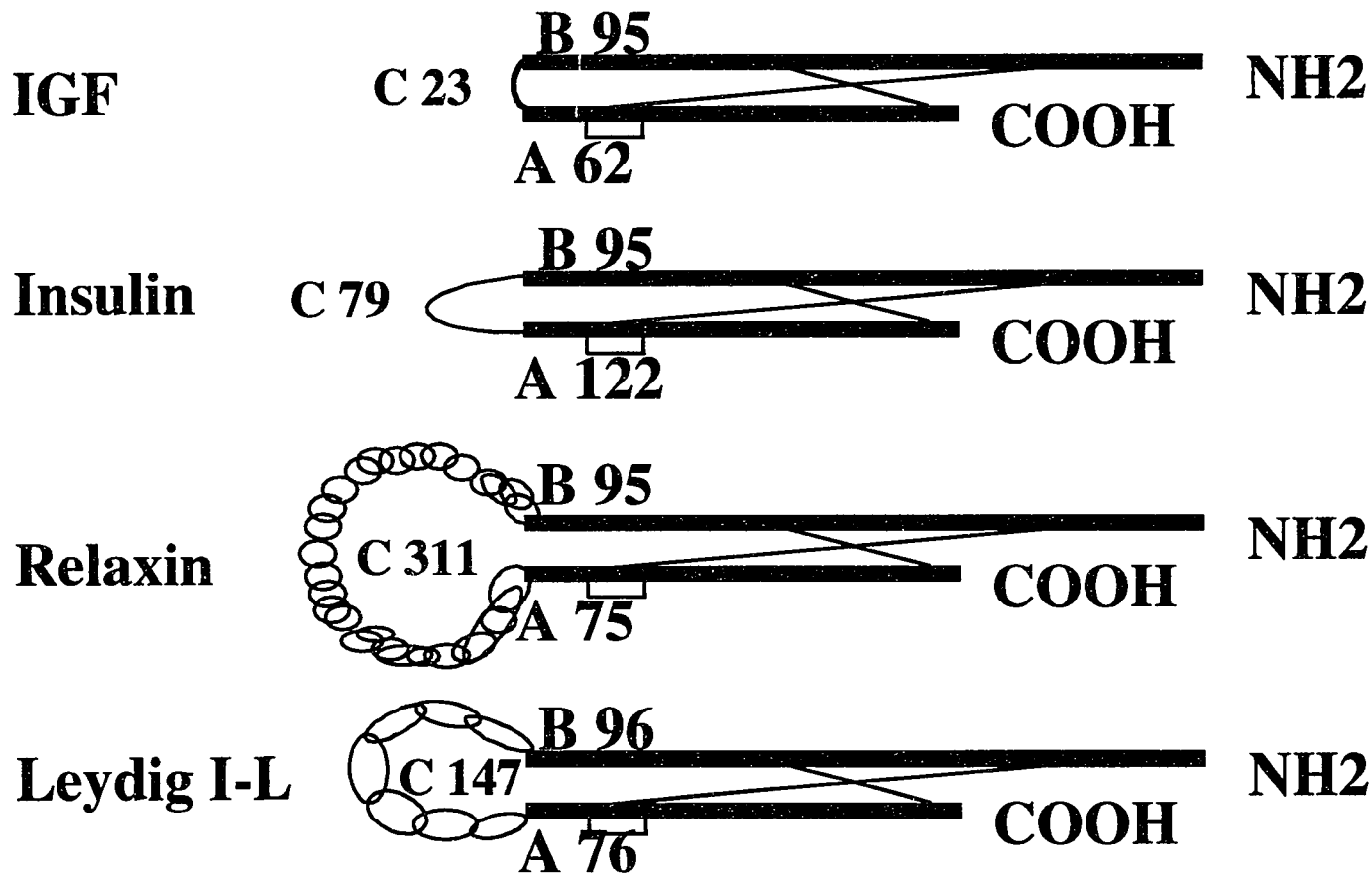


Figure 6 Schematic illustration of prohormone structures for IGF, Insulin, Relaxin and Ledig I-L adopted from Kemp and Niall, Relaxin, Viat. Horm 1984

and there is little or no crossreactivity of RLX with insulin or IGF receptors (Adashi, et al., 1988). This is not unexpected, given that the amino acids responsible for insulin bioactivity are absent in relaxin and the amino acids responsible for relaxin bioactivity are absent in insulin (Bullesbachand Schwabe, 1994).

1. Relaxin levels in pregnancy

Maternal serum RLX levels rise rapidly in a conceptus cycle parallel with human chorionic gonadotropin (HCG) (Stewart, et al., 1990). Systemic RLX produced by the corpus luteum peaks the first trimester of pregnancy and thereafter gradually declines to term (44) Fig 7. Relaxin is produced by the glandular epithelium in the follicular phase of the menstrual cycle and after ovulation, the decidualized stromal cells of this tissue also contribute to its production (Bryant-Greenwood, et al., 1993). It is these cells which continue to produce the hormone into early through late pregnancy (Bryant-Greenwood et al., 1993). As the placental mass increases the production of RLX from the syncytiotrophoblastalso increase (Sakbun, et al., 1990; Bogic, et al., 1995), but neither of these serves to appear to contribute to the levels of RLX in the circulation.

Several laboratories demonstrated concomitantly the presence of RLX in the human decidua and placenta using isolation and bioassay Bigazzi et al., 1982, Fields and Larkin, 1981 isolation and RIA Yamamoto et al., 1982. The unequivocal demonstration that RLX is a product of these tissues required sensitive molecular techniques, thus RT-PCR (Hansell, et al., 1991) showed the presence of both RLX H1 and H2 in decidua and placental trophoblast and in situ hybridization (Bogic, et al., 1995) showed the presence of human relaxins in decidua and the syncytiotrophoblasts of the villious trophoblast.

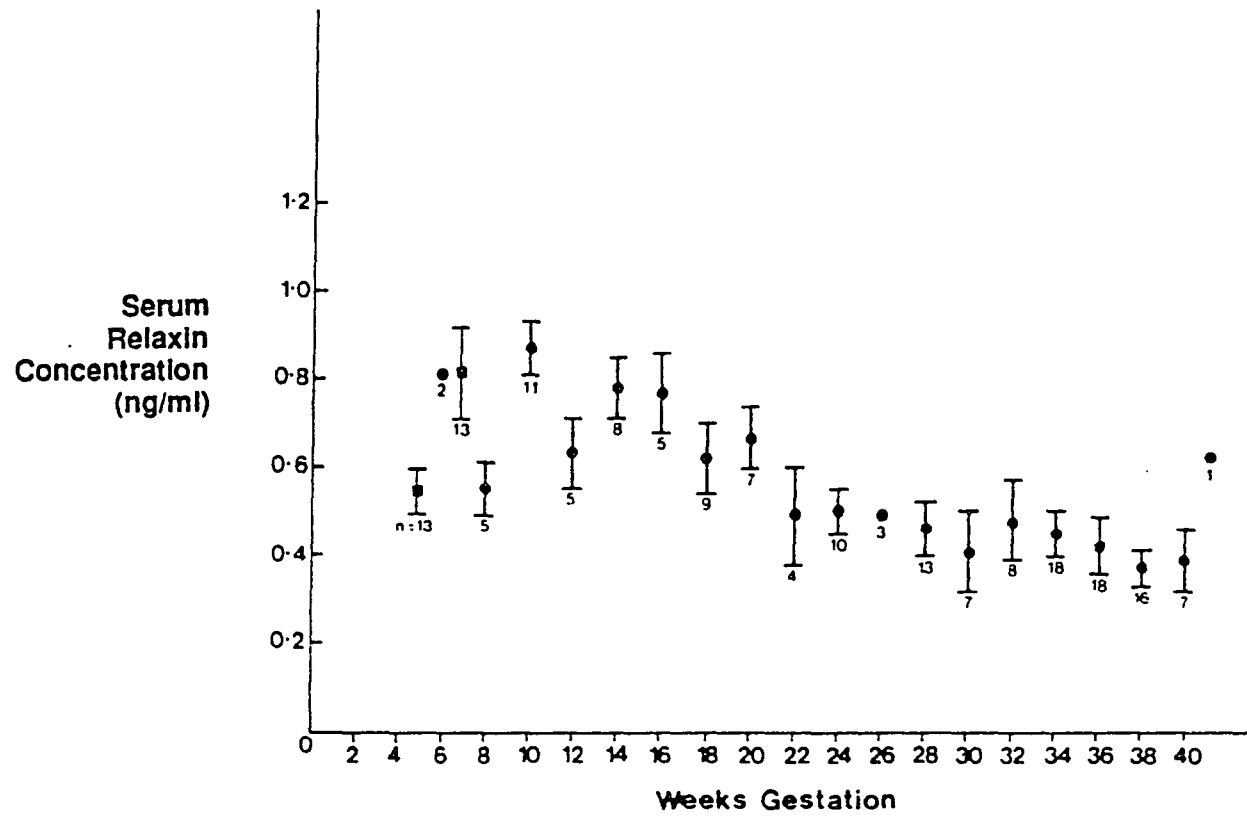


Figure 7. Serum levels of relaxin throughout gestation

adopted from Bell et al., 1987

2. Physiological roles of relaxins

RLX has two classical biological activities; to cause the rapid inhibition of myometrial activity and to induce a slow change in the organization of connective tissue at its target sites (Krantz, et al., 1950; Koay, et al., 1986). Pubic ligament separation in women is identifiable by the end of the first month of pregnancy, becomes maximal by the end of the fifth to seventh month and remains relatively constant during the final trimester coinciding temporally with maternal serum RLX levels (Thoms, 1936). However, the maintenance of pregnancy and subsequent parturition can occur normally in the absence of a corpus luteum, as seen in women with ovarian failure who become pregnant by egg donation (Johnson, et al., 1981). These observations led Dr. Bryant-Greenwood to propose that decidual/placental RLXs are autocrine/paracrine hormones of greater biological significance to the synchronization of human parturition pregnancy than systemic corpus luteum derived RLX (Koay, et al., 1985; Sakbun, et al., 1990; Hansell, et al., 1991)

As pregnancy advances the amnion and chorion become contiguous with the decidua, allowing complex autocrine/paracrine interactions as well as an influence on and by their pericellular and extra cellular matrices. The human fetal membranes are highly collagenous structures (refer to section 1.1) which during the last weeks of pregnancy lose small amounts of collagen in order to be able to stretch under the increasing tension created by the rapid growth of the fetus and placenta (Skinner et al., 1981). It is this controlled loss of collagen that permits the membranes to stretch and possibly prevents the premature rupture of membranes (Skinner, et al., 1981). This hypothesis was tested

almost a decade ago when only porcine relaxin was available, using short term primary cultures of dispersed amnion and chorion cells, it was shown that RLX stimulated the secretion of plasminogen activator (collagenolytic activity) into the media of these cells (Koay, et al., 1986). This has recently been repeated using synthetic human RLX (H2) on amnion, chorion and decidual cell explants. It was demonstrated that RLX causes a dose related increase in the expression of proMMP-1 and proMMP-3 but not proMMP-2 or TIMP-1 (tissue inhibitor of MMPs) in these tissues with concomitant increase in the level of MMP-1, MMP-3 and MMP-9 activities in the medium of these explants (Qin *et al.*, submitted).

3. Summary

The maternal decidua and fetal placenta have been shown to transcribe both the human RLX genes H1 and H2. PRL on the other hand is a product of the maternal decidua only. The same decidual cells appear to be responsible for the production of these three hormones. Autocrine/paracrine action of RLX on these cells has been suggested by the increased secretion of PRL on the addition of RLX to decidual cell cultures. This work implies the presence of a RLX R on the decidual cells, similar work on the addition of PRL to these cells has not been carried out.

The gene and derived protein structure of the prolactin receptor (PRL-R) are known, whereas little is known of either RLX-R in any species. On the other hand, a well defined biological action of RLX on the decidua and chorion leave to other gene transcription of proMMP-1 and proMMP-3 has been demonstrated, whereas the actions of PRL on either the decidua or chorion cells or the placental trophoblast are unknown.

C. Prolactin and Relaxin Receptors

1. The PRL receptor

The initial step in the mechanism of action of a peptide hormone is the binding of the ligand to its cell surface receptor. Hormone receptors are defined as protein molecules with low capacity, high affinity and high specificity for a given ligand, the binding of which results in a biological response/action (Kelly, et al., 1991).

The rat PRL-R was purified using immunoaffinity chromatography on solubilized estrogen-treated female rat liver membrane preparations. Preparative amounts were partially purified by electroelution using SDS PAGE, this partially purified receptor was digested with trypsin, the resulting peptides were further purified by reverse phase HPLC. The peptides were then sequenced by gas phase sequence analysis. Oligonucleotide probes were prepared and used to screen an estrogen primed polysome enriched λ gt11 library (Boutin, et al., 1988). The rat PRL-R cDNA probe was then used to probe a human hepatoma library and a T47D breast cancer library (Boutin, et al., 1989). The resulting amino acid sequence showed striking similarities to the human growth hormone receptor (GH-R) (Boutin, et al., 1989). This marked resemblance is not surprising considering the close homology between the hormones themselves (Niall, et al., 1971; Kelly et al., 1991). The human PRL -R is composed of 598 amino acids in its mature form and is classified as a member of the now larger super-family of the GH, PRL and cytokine receptor super family (Fig 8).

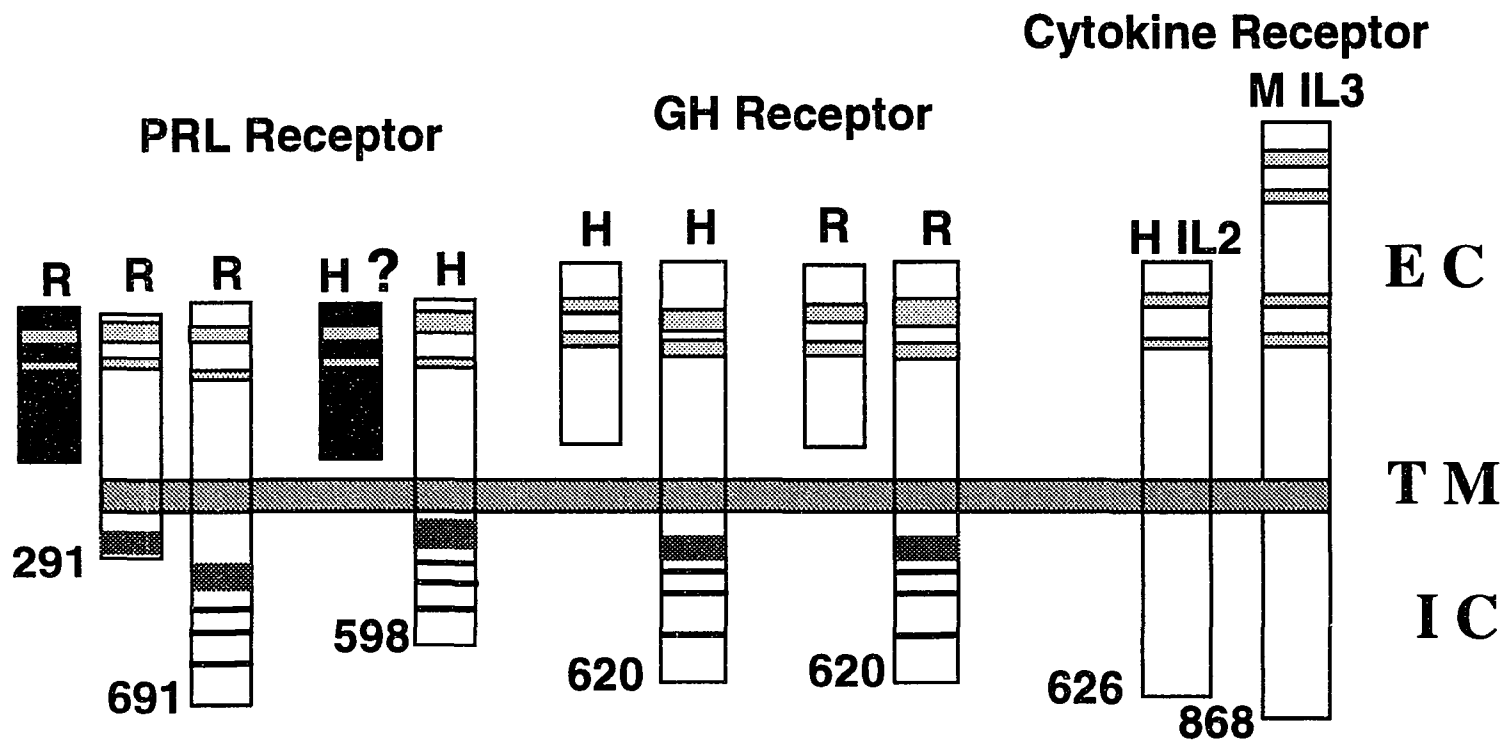


Figure 8. Schematic illustration of the growth hormone, prolactin, and cytokine receptor family
 EC: extracellular, TM: transmembrane, IC: intracellular domains, H: human, M: mouse, R:rat
 H?: not confirmed Mercado and Bauman, 1994

A single form of the PRL receptor (67 kDa non glycosylated), is the only form yet fully defined (Boutin, *et al.*, 1989). Multiple forms of the PRL receptor have been identified in the rat and rabbit ranging from 90 kDa to 16 kDa (Buczko, *et al.*, 1989; Sugiyama, *et al.*, 1994). Recently a soluble PRL/GH 'binding protein' has been identified in human milk but is not yet fully characterized (Mercado and Bouman, 1994). Figure 8 summarizes this data and shows that both the PRL and GH receptors are divided into two receptor classes: the short and long forms (Kelly *et al.*, 1991). Both receptors are composed of a large extracellular domain (peptide binding region), a single transmembrane segment and a cytoplasmic domain that varies in length (depending on the class of receptor). The human PRL-R gene is located on the short arm of chromosome 5p13--14, which also bears the GH-R gene (5p13.1--p12) (Barton, *et al.*, 1989; Arden, *et al.*, 1990). It is likely that by analogy to the hormones themselves gene duplication led to the evolution of two structurally related but distinct receptors for GH and PRL. There are three features that are prevalent throughout the members of this receptor superfamily; (1) the presence of two pairs of cysteines located toward the N terminal region of the peptide; (2) a highly conserved WSxWS motif, usually just flanking the transmembrane domain (with the exception of GH which lacks the repeat); (3) a duplication of the extracellular region into two homologous immunoglobulin-like subdomains of approximately 100 amino acids. The pairs of cysteines appear to be involved in the binding of PRL to its receptor, since site directed mutagenesis of any of these cysteines abolished the binding of PRL (Rozakis-Adcock, *et al.*, 1991; 1992). The WS motif is required for high affinity

binding of PRL and GH, it has been proposed that this motif serves as a target site for accessory protein association, which maybe essential for the creation of high affinity binding sites (Rozakis-Adcock, et al., 1992).

Human experimentation is limited and information concerning both PRL and RLX receptors in the human is scarce. Therefore, it is necessary to discuss selected nonhuman data and where available data on the human will be addressed.

In the rat, the short form of the PRL receptor is encoded by a 1.8 kb transcript only and the long form by three transcripts of 2.5, 3.0 and 5.5 kb (Shirota, et al., 1990). These different mRNA transcripts are products of alternative splicing of a primary transcript (Kelly, et al., 1991). Guillaumont and Cohen demonstrated the presence of four different molecular forms (80, 50, 40 and 16 kDa) of the PRL receptor protein at different physiological stages in the female rat (Guillaumot, et al., 1994). The 16 kDa form is found only in the lactating mammary gland and is postulated to be a product of inducible enzymatic metabolism (Guillaumot, et al., 1994). Postel-Vinay et al., 1991, demonstrated soluble forms of the PRL receptor in rabbit milk of 55 and 33 kDa . These and the 16 kDa protein in the rat mammary gland probably correspond to the extracellular binding domain of the PRL receptor, given that monoclonal antibodies specific to the extracellular domain of the receptor recognize the soluble forms of the PRL receptor and I¹²⁵ ovine PRL binds to and is competed off by the 16 kDa form (Guillaumot, et al., 1994; Postel-Vinay et al., 1991).

The significance of the multiple transcripts and multiple forms of the PRL receptor is not known at this time, however, in the rat there is evidence that tissue specific

hormonal regulators control the expression of the different mRNA transcripts and ensuing translated receptors (Sugiyama, et al., 1994). The human PRL receptor has not been extensively explored.

The mature membrane bound human PRL-R is a member of the second receptor class (long form) which differs by addition of approximately 207 amino acids in the cytoplasmic domain. Recently a PRL/GH binding protein in human milk has been partially characterized (Mercado and Bauman, 1994), thus possibly adding a class to the human PRL-R. The mRNA transcripts for the human PRL-R have been shown to be 2.3, 3.5 and 7.3 kb in a breast cancer cell line (T47-D), breast cancer biopsies and in the chorion leave. These transcripts plus an additional transcript of 10.5kb was detected in the MCF-7 breast cancer cells treated with estrogen (Boutin, *et al.*, 1989; Ormandy, *et al.*, 1990). It is not known whether the different human mRNA transcripts arise from post-translational modifications and/or alternative splicing. Mercado and Bauman characterized a 30 kDa GH/PRL binding protein in human milk. The hormone binding specificity for this novel binding protein is consistent with that of the PRL-R, as both GH and PRL will bind to this receptor but only GH will bind to the GH-R (Mercado and Bauman, 1994).

2. The relaxin receptor

There is a high degree of sequence heterology (55%) between the RLXs of closely related species (Schwabe, 1993; Bryant-Greenwood and Schwabe, 1994). The finding that all mammalian RLXs determined thus far are biologically active in the rat implies the presence of a specific conserved region(s) on the RLX molecule that interacts with the RLX receptor(s) (Bullesbach, Schwabe, 1993). All true RLXs have the following

conserved B chain residues: B11 cys, B12 gly, B13 arg, B17 arg, B23 cys and B24 gly (numbering based on human RLX H2). The arginine residues at positions B13 and B17 have been shown to be essential for biological activity (Bullesback, Schwabe, 1988). Although arginines characteristically are not considered active site residues, the projection of this position on the molecular surface as well as the conservation of these residues (but not position in the B chain) in all known RLXs implicates the necessity of the arginines for receptor binding (Bullesback, Schwabe, 1988; Bullesback, et al., 1992). It has been suggested that the binding of RLX to its receptor is mediated by “a two prong positive charge and hydrogen-binding interaction via the B13 and B17 arginines” (Bullesback, et al., 1992) likewise, this binding mechanism appears to be unique in the hormone receptor interaction field (Bullesback, et al., 1992). The A chain of relaxin also contains several conserved amino acids: 1.A9 lys, 2.A14 gly, 3.A17-18, lys/arg, 3.A22 lys/arg. These amino acids confer appropriate conformation essential for binding of RLX to its receptor and hence its biological activity while the B chain of RLX appears to contain the receptor interaction site and therefore mediates its biological response (Bullesback and Schwabe, 1994; Bullesback and Schwabe, 1988).

The RLX receptor has been partially characterized in a variety of tissues over a period of 25 years using I¹²⁵ labelled and ³²P labeled relaxins; rat myometrium (Mercado-Simmen, et al., 1982), pig myometrium and cervix (Mercado-Simmen et al., 1982) human fetal membranes (Koay, et al., 1986), rat brain (Osheroff, et al., 1991), and rat heart atrium (Osheroff, et al., 1992). Two labeling methods have been used for the identification and characterization of the RLX receptor in a number of species; ¹²⁵I labeled porcine

RLX, which utilizes a tyrosine residue for iodination and 32 P γ ATP labeled human H2 RLX, which phosphorylates serine and threonine residues using a cAMP-dependent protein kinase. Since human RLX H2 contains only a single tyrosine positioned near the N-terminal on the A chain (refer to Fig. 5), iodination proved to be unsuccessful and alternative methods were sought. The phosphorylation of human immune interferon was first reported in 1986 by Kung and Bekesi. This was then adopted for the labeling of human RLX H2 (Osheroff, et al., 1990).

The first detailed characterization of the RLX receptor was performed on the rat uterus by Mercado-Simmen, et al., 1980. Her work demonstrated that binding of 125 I porcine RLX to the rat myometrium receptor is time, temperature, cation, pH and estrogen dependent (Mercado-Simmen, et al., 1980). In addition, Mercado-Simmen et al. showed a significant down regulation of the RLX receptors with an acute dose of RLX *in vivo* (Mercado-Simmen, et al., 1980). She also demonstrated that the myometrium and cervix of cyclic prepubertal domestic pigs pretreated with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) exhibited high affinity binding sites for the labeled hormone (Mercado-Simmen, et al., 1982). Ten days postovariectomy, the porcine myometrium exhibited a significant decrease in the number of RLX receptors and by day 16 postovariectomy, the receptor levels had decreased to undetectable levels. Estrogen treatment (over a period of seven days), recovered these myometrial receptors to preovariectomy cyclic levels (Mercado-Simmen, et al., 1982). However, this recovery with estrogen treatment was not seen in the cervical RLX receptors. This data suggests a tissue specific hormonal control for the expression of the

RLX receptor in the pig myometrium and cervix (Mercado-Simmen, et al., 1982). This is not unreasonable since the biological roles of relaxin on these tissues causes myometrial quiescence and cervical dilatation, events that require separate modulation for the coordination of peripartal events (Mercado-Simmen, et al., 1982; Bryant-Greenwood, Schwabe, 1994). Koay et al., 1986, established the presence of the RLX receptor in the human fetal membranes using ^{125}I labeled porcine RLX with membrane enriched fractions of human fetal membranes. She also demonstrated a biological response; RLX added to cultured human amnion/chorion cells caused an increase in the release of plasminogen activator and collagenolytic activity into the media, lending additional support for the presence of a RLX receptor in these tissues(Koay et al., 1986).

The chemical synthesis and subsequent ^{32}P labeling of human RLX H2 made it possible for the first time to study the properties and interactions of human RLX and its homologous receptor (Fig. 5). However, studies with this ligand were conducted in the rat using cryosectioned tissues from the uterus, cervix and brain (Osheroff, et al., 1991). The presence of the RLX receptors in the rat brain was unexpected, however their specificity and affinity were shown to be similar to those in the rat uterus and cervix (Osheroff, et al., 1991; Osheroff et al., 1991). Using this methodology a single class of receptor binding sites was characterized in the neocortex and subfornical organ of the rat brain, interestingly separate studies using in situ hybridization demonstrated the presence of mRNA for RLX in this tissue making the brain a separate autocrine paracrine system (Osheroff and Ho, 1993). These results taken together show an autocrine/paracrine system operating behind the blood brain barrier.

D. Statement of problems

The mechanisms responsible for the onset of spontaneous human parturition at term are not known. It has taken over 20 years of basic research to recognize that normal parturition in the human is controlled differently from other species, albeit with the same complement of hormones (Liggins, 1985; MacDonald, 1993). The thesis that the endocrine controls are localized within the human as opposed to control by systemic signals in other species is becoming increasingly recognized. PRL was one of the first such endocrine hormones from the pituitary recognized as a product of maternal decidua (Golander *et al.*, 1978). Similarly, because the classical source of human RLX the ovaries of pregnant women, were not available for its isolation (Bryant-Greenwood, 1982), a more accessible tissue was studied by serendipity and the decidua/basal plate was shown to contain relaxin (Yamamoto and Bryant-Greenwood, 1981). This was proposed to act locally in an autocrine/paracrine mode (Koay *et al.*, 1983, '86). Since then, oxytocin has been shown in the human to be produced in the fetal membranes, thus together with local PRL and RLX may be more significant in the events of human parturition than their systemic homologues from the hypothalamus, pituitary and ovary respectively.

The theme of this dissertation is to examine the production and distribution of PRL and RLX receptors in the human fetal membranes, decidua and placenta at accessible times in the peripartal period. This should provide further insight into their autocrine/paracrine roles in peripartal processes.

1. Prolactin receptor

The first question addressed in this project was to define the cellular targets of PRL in the amnion, chorion, decidua and placenta and to examine its transcription and translation in relationship to parturition.

In order to determine the sites of translation of the hPRL-R gene, quantitative Northern analysis has been used on tissues at different time points in the peripartum with a human PRL-R cDNA probe. This probe was kindly provided to our laboratory by Dr. P.A. Kelly (INSERM Unite'344). After defining the tissues transcribing the PRL-R its cellular localization was examined further by in situ hybridization by two 48mer synthetic oligo probes corresponding in sequence to segments of the extra and intracellular domains of the PRL-R. The cDNA probe was initially used for the preparation of a cRNA probe. However, specificity problems made it necessary to prepare and use the two 48mer oligo probes. The translated PRL-R protein was localized using a monoclonal antibody made to the extracellular domain of the rat PRL-R, (U5, kindly provided to our laboratory by Dr. P.A. Kelly, INSERM, Unite'344), and the avidin-biotin immunohistochemical technique. Western blot analysis was also performed on ACD lysates and media from ACD explants after 48 hours of incubation.

2. Relaxin receptor

This laboratory identified specific relaxin receptors in membrane enriched fractions from dispersed human amnion and chorion cells using I¹²⁵ labeled porcine relaxin (Koay et al., 1986). The aim was to extend by using the synthetic human relaxins H1 and H2 (A24, B33), for characterization of the human RLX-R(s) in membrane enriched

preparations of amnion, chorion and decidua tissue. To determine the time in the peripartal period when the tissues have the greatest amount of RLX binding, it was necessary to survey the time points available to us at term (C-section, emergency C-section and normal labor and delivery). Term C-section membranes appeared to have the highest capacity of binding for the labeled human relaxins. A number of parameters that could effect RLX binding to its receptor had to be examined:

- Freezing of membrane fractions
- Labeled RLX fractions off the HPLC
- Time
- Temperature
- pH
- Protein concentration (per reaction tube)

Establishment of these conditions allowed the binding comparisons of H1 and H2, in addition to the displacement ability of human H1, H2, porcine and guinea pig relaxins and insulin as discussed in chapter 2 of this thesis.

Experimentally, the major problem in working with any relaxin is its hydrophobic nature, as discussed extensively in Problems In The Study Of Receptors For Relaxin (Greenwood et al., 1981). The following procedures were used when working with RLX in attempt to minimize its adsorption to surfaces.:

- polyether-ether-ketone tubing and/or titanium frits for the components for the HPLC.
- tubes and pipette tips were made of polypropylene pretreated of all tubes and pipette tips with silicone

E. Organization of the dissertation and allocation of contribution

The body of this dissertation is on the PRL-R submitted to the Journal of Clinical Endocrinology and Metabolism and on the RLX-R , currently in press.

* An overview of the tissues, hormones and their receptors involved in this work, not fully described in these papers, is presented in Chapter I. Since the two papers were published in different journals, the format for each one is slightly different. Although there are references at the end of each chapter, the bibliography of the entire dissertation is listed alphabetically in the last chapter.

*The title of chapter II is THE HUMAN PROLACTIN RECEPTOR IN THE FETAL MEMBRANES, DECIDUA AND PLACENTA. My contribution as first author of this paper included mRNA extraction, Northern hybridization and quantitation, in situ hybridization, lysate and Western blot analysis. In addition I was responsible for approximately 50% of the manuscript preparation.

* The title of chapter III is CHARACTERISTICS OF THE BINDING OF ³²P-LABELED HUMAN RELAXINS TO THE HUMAN FETAL MEMBRANES. This is a joint first authorship paper. I was responsible for the labeling of the RLX peptide (both H1 and H2). All binding assays and subsequent assessment of data were performed jointly.

CHAPTER II

The Human Prolactin Receptor in the Fetal Membranes, Decidua and Placenta

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A. Abstract

Human prolactin (PRL) is synthesized and secreted by the maternal decidua but not by the chorionic cytotrophoblast of the chorion laeve or the placenta. The site(s) of action for decidual PRL are currently unknown. Accordingly, Northern analysis and in situ hybridization histochemistry have been used respectively to quantitate and localize the expression of the prolactin receptor (PRL-R) gene within the uterus during the peripartal period. Immunocytochemistry and Western blot analysis using an anti-PRL-R antibody (U5) localized the translated protein at the cellular level in the same tissues.

As judged by the level of expression of the PRL-R gene and its translated products, the chorionic cytotrophoblast has been shown to be a primary site of action. Novel sites were also shown in the decidua, placental trophoblast and the amniotic epithelium. In situ hybridization was not obtained in the latter despite positive Northern analysis and immunostaining.

Western analyses with an antibody (U5) to the extracellular domain of the rat PRL-R detected six major molecular species of 95, 85, 63, <63, >30 and 30 kDa in cytosol from separated amnion, chorion and decidua. The two bands at 95 and 85 kDa were approximate values only and represent the mature glycosylated forms of the human PRL-R. The other four major bands were partial degradation products from the PRL-R showing tissue specific processing and patient to patient variation related to the spectrum of proteases present in these tissues. The 63 and 30 kDa PRL-R related proteins were detected in both the cytosol and media from amnion, chorion and decidua and were present in amniotic fluid. The 30 kDa species was equal in size to a recently reported PRL binding protein in human milk and their release into amniotic fluid suggests possible function(s) as binding and or/PRL transport

proteins in these tissues. The >30 kDa species was detected in high amounts in both cytosol and media from the decidua but was absent from amniotic fluid. Further work is required to clarify the structural relationships and potential functions of these immunologically PRL-R related proteins.

An increased expression of the PRL-R during labor and delivery in fetal membranes and placenta was shown and strongly supports a role for decidual PRL acting as an autocrine factor in the decidua and as a paracrine factor in the amnion, chorion and placenta.

B. Introduction

PRL was the first of many hormones recognized as products of the human decidualized endometrium (1). In situ hybridization histochemistry has demonstrated unequivocally that the decidua indeed is the major source of PRL within the uterus (2,3). The net synthesis of decidual PRL has been demonstrated in vitro using decidual cells and explants (4,5). However human PRL has a single gene transcribed in both the pituitary and decidua but differing in the 5'UTR in these tissues (6-8). The additional exon in the decidual PRL cDNA is responsible for its unique regulation from this tissue.

Binding studies for hPRL have been conducted with chorio-decidua and breast tissues (9-11). A receptor from chorio-decidua has been partially characterized and shown to have minimum molecular weight for its binding domain of approximately 40,000 daltons with general characteristics of lactogenic hormone receptors in general (11). The structure of this receptor was further elucidated by screening cDNA libraries from human hepatoma cells and a human breast cancer cell line, using the cDNA for the rat PRL-R as a probe (12). A long form of the human PRL-R has been identified which contains 598 amino acids in its mature form,

whereas both a long and a short form of the PRL-R have been identified in other species (13).

Recently a potential growth hormone/PRL binding protein has been identified in human milk but the precise nature and function of this protein remains to be defined (14).

The physiological role(s) of PRL within the maternal-fetal-placental compartment are unknown. It has been proposed from studies conducted with rhesus monkeys that PRL has an osmoregulatory function (15). The distribution of the targets of decidual PRL would further our understanding by allowing the spatial and temporal localization and quantitation of the receptor in the fetal membranes/decidua and placenta. This approach has recently been made available time points, now made possible by the availability of the PRL-R cDNA (12) as well as antibodies which recognize the PRL-R (16-17).

C. Materials and Methods

Placentas with attached fetal membranes and decidua from elective pregnancy terminations between 12.5-20 weeks gestation (n=7), were kindly provided by Dr. M.L. Casey and colleagues, Dept. of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, Dallas, TX. The tissues were placed on ice for 30-120 min prior to dissection, frozen in liquid nitrogen, stored at -80 C, shipped to Hawaii on dry ice and again stored at -80 C until use.

Tissues from unselected preterm deliveries at 30-35 weeks gestation, group 1 (n=6) were collected with patients' consent by Drs. Naomi Wahl and Lynnae Millar at the Kapiolani Medical Center for Women and Children, Honolulu, HI. All term tissues were collected at this hospital and brought to the laboratory on ice, within 1h of removal or delivery. Elective term

Cesarean section tissues (38-41 weeks gestation), were from patients having repeat sections and before labor, group 2 (n=18), or in labor, due to medical emergencies arising during the labor process, group 3 (n=5). Tissue was also collected from women undergoing normal labor and delivery at term, 38-42 weeks gestation, group 4 (n=17). Tissues were collected with informed consent and under appropriate protocols approved by the institutional review boards of Universities and Kapiolani Medical Center.

Chorio-decidua and placental trophoblast samples were obtained by stripping the amnion from the chorio-decidua and cutting the basal plate from the placenta. Further dissection of the decidua required scraping the decidua from the chorion-laeve and for the villous trophoblast, excising a region from the central area of the placenta. In the separated cellular layers amnion, decidua and basal plate were pooled from more than one patient in order to obtain enough total RNA for the isolation of poly (A)⁺ RNA, while chorion and villous trophoblast were pooled from patients for consistency. All tissues, amnion (N=8), chorion (N=3), decidua (N=5), villous placenta (N=3) and placental basal plate (N=5) were dissected, frozen in liquid nitrogen and stored at -80 C until use.

Probes

The human PRL-R cDNA probe (12) was excised from a Bluescript vector by digestion with PstI (New England Biolabs, Beverly, MA) at 37 C for 2h releasing a 1.35 kilobase pair cDNA fragment, the position of this probe is shown in Fig 1. The PRL cDNA probe (712 base pair) was a generous gift from Dr. Nancy Cooke, University of Pennsylvania (6) and the human glyceraldehyde 3 phosphate dehydrogenase (G3PDH) probe was purchased from Clontech Inc., Palo Alto, CA. Two 48 mer oligo probes were designed by a probe selection program

(Gene Runner: Hastings Software Inc., N.Y., NY), for the extracellular and intracellular domains of the human PRL-R and made by the Biotechnology Molecular Biology Instrumentation Facility, University of Hawaii, using the phosphoramidite method on an Applied Biosystems DNA Synthesizer model 380B (Foster City, CA). The extracellular probe corresponds to nucleic acids 40-88, and the intracellular probe to nucleic acids 2261-2308 according to the numbering used by Boutin *et al* (12). Their sequences and positions are shown diagrammatically in Fig 1.

Labeling

The PRL-R cDNA, PRL cDNA and G3PDH cDNA probes were labeled with [$\alpha^{32}\text{P}$] dATP and the random primer labeling kit (GIBCO BRL, Grand Island, NY). The labeled probe was separated from unincorporated radioactivity on a Sephadex G50 spin column (Pharmacia, Piscataway, NJ) followed by precipitation with ammonium acetate (0.5 X vol. of 7.5M ammonium acetate and 2.2 X vol. of 100% ETOH) for 2h at -20 C. The oligo probes were 3'end-labeled (tailed) by terminal deoxynucleotidyl transferase (US Biochemical corp, Cleveland, OH). Briefly, 5 pmol of oligoprobe and 50uCi of [^{35}S]dATP or [$\alpha^{32}\text{P}$]dATP (NEN Research Products, Boston, MA) were incubated in 50 μl of reaction mixture containing 10 μl of 5X tailing buffer and ~20U of terminal deoxynucleotidyl transferase (TdT). Following incubation at 37 C for 1h non-incorporated radioactive nucleotides were removed by Sephadex G25 spin column chromatography (Pharmacia Piscataway, NJ).

Northern blot analysis

All reagents were of the highest quality available and all equipment was treated using standard techniques to eliminate RNase activity. Solutions were treated with diethylpyrocarbonate (Sigma, St Louis, MO) at a final concentration of 0.1% and autoclaved. Total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (18). Poly (A)⁺ RNA was isolated by affinity chromatography on oligo (dT) cellulose (GIBCO BRL, Grand Island, NY), (19). Poly (A)⁺ RNA fractions were stored at -20 C in 70% ethanol until use.

For Northern blotting, Poly (A)⁺ RNA (20 µg) was denatured using 1mol/L glyoxal and electrophoresed on a 1.4% agarose gel (BioRad Laboratories, Hercules, CA) buffered with 0.01mol/L phosphate buffer, pH 6.5. A DNA ladder (λ phage DNA, *Hind III* digest, New England Biolabs, Beverly, MA) and a 0.16-1.77 kb RNA ladder (GIBCO BRL, Grand Island, NY) were used as molecular size markers. The gel was stained with 0.5 µg/ml ethidium bromide for 20 min and photographed under UV light. RNA was transferred from agarose gels to Magnagraph 0.45µm nylon membranes (MSI, Westborough, MA) in 10X SSPE (1X SSPE= NaCl 8.7g/L, NaH₂PO₄·H₂O, 1.4g/L, EDTA 0.37 g/L, NaOH to pH 7.7) by capillary transfer overnight. The membranes were then rinsed in 5X SSPE and baked at 80 C for 1h. The filters were stored in sealed containers at 4 C until use. The membranes were prehybridized at 42 C in a solution containing 50% formamide, 5X SSPE, 5X Denhardt's solution (0.1% ficoll, 0.1% polyvinyl-pyrrolidone, 0.1% bovine serum albumin, 0.1% sodium

dodecyl sulfate (SDS), and 100 μ g/ml denatured salmon sperm DNA for 3 to 4 h. Hybridization of the mRNA was then carried out at 42 C for 18 h in 50% formamide, 5X SSPE, 5X Denhardt's solution, 5% dextran sulfate, 0.1% SDS, 100 μ g/ml denatured ssDNA and the labeled probe at 5×10^6 cpm/ml. After hybridization, the filters were washed twice in 6X SSPE, 0.1% SDS at room temperature for 15 min each and once in 3X SSPE, 0.1% SDS at 65 C for 30 min and finally in 1X SSPE for 15 min at room temperature, exposed to Fuji RX film (Fuji Photo Film, CA Ltd., Kanagawa, Japan) with intensifying screens at -20 C for 24 h. Northern blots were deprobed using 50% formamide, 3X SSPE at 65 C for 1 h, filters were reprobed with the cDNA for prolactin and exposed for 8 h and finally deprobed and reprobed with G3PDH labeled and exposed for 30 min. The latter was used as a control for the amount of poly (A)⁺ RNA loaded.

Filters were quantitated by scanning densitometry using a Biorad model 620 Video Densitometer with a 1-d Analyst software package for IBM-PC. Hybridization signal intensity was expressed as the area under the assigned intensity peak. In order to correct for the amount of poly (A)⁺ RNA loaded on the filters, each filter was hybridized with the human G3PDH cDNA. Because the expression of G3PDH varied with the different tissues it was not possible to use it as an inter-tissue standard. It was used to standardize the mRNA within a single group of tissues. Statistical analysis was by one-way analysis of variance followed by Tukey-Kramer multiple comparison test.

In Situ hybridization

Tissues were cryosectioned (10-20 μm) at -20 C , thaw-mounted onto Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at -80 C . Prior to hybridization, sections were warmed to room temperature, prefixed in sterile phosphate buffer (PBS, 0.015mol/L pH 7.6) containing 4% paraformaldehyde, rinsed twice in PBS, incubated for 10 min with acetic anhydride 0.25% freshly prepared in 0.1mol/L triethanolamine (TEA) HCL/0.9% saline, pH 8.0. Sections were serially dehydrated in ethanol, defatted for 5 min in absolute chloroform, rehydrated in graded ethanols and air-dried. Trophoblast specimens were pretreated after fixation with proteinase K (Boehringer Mannheim, Indianapolis, IN) $1\mu\text{g/ml}$ in 50mmol/L Tris HCL (pH 7.5) containing 5mmol/L EDTA (pH 8.0) in a 37 C water-bath for 30 min. After digestion, slides were quickly rinsed in DEPC treated water at 25 C followed by a 2 min rinse in 0.1mol/L TEA (pH 8.0). Acetylation and dehydration steps as per the non-treated slides followed proteinase K treatment. The hybridization procedure was based on methods described by Young *et al*, (20). The [$\alpha^{35}\text{S}$]-labeled probes were dissolved in a hybridization buffer containing 50% formamide, 4 X SSC, sheared ssDNA (500 $\mu\text{g/ml}$), yeast tRNA (250 $\mu\text{g/ml}$), 1X Denhardt's solution and 10% dextran sulfate. The oligo probe mixture was applied to each slide (total volume 100 μl /slide). Preliminary dilutions of labeled oligo probes showed that $1-2 \times 10^8$ cpm/ml of hybridization solution give a good signal with low background. Slides were covered with glass coverslips (Fisher Scientific, Pittsburgh PA) and incubated in a humidified chamber at 37 C overnight. Following incubation the slides were

rinsed in 1 X SSC at room temperature to loosen and remove coverslips and were washed 4 times in 2 X SSC/50% formamide at 40 C for 15 min, then twice in 1 X SSC for 30 min and air dried in vertical slide racks, all at room temperature. Slides were dipped in NTB-2 liquid photographic emulsion (Eastman Kodak; diluted 1:1 with distilled water) and exposed at -20 C for 14 days. After development, they were stained with toluidine blue (0.5%) for 30-60 seconds and photographed under both bright and darkfield illumination with a Zeiss photomicroscope II, (Zeiss, Oberkochen, Germany).

To demonstrate the specificity of the 48mer oligo probes, Northern analysis was carried out with mRNAs from chorio-decidua and placental trophoblast. The results using the antisense 48mer oligo probes were the same as those obtained using the PRL-R 1350 bp cDNA probe (data not shown). The sense probes, on the other hand, as expected, failed to hybridize (data not shown). These results validated the 48-mer oligo probes prepared.

Immunocytochemistry

Placenta with attached membranes were obtained from women at term gestation: Cesarean section (without labor, n=5) and after normal spontaneous delivery (n=5) at Kapiolani Medical Center for Women and Children (Honolulu, HI) within 1h of delivery. Representative slices (10mm thick) were cut across the placenta and basal plate and 1 cm pieces of the fetal membranes with adherent decidua were dissected. The samples were immediately fixed in Bouin's solution (16-24 h at 25 C) and processed for paraffin embedding. Seven-micron sections were cut and mounted on Vectabond (Vector Labs Inc., Burlingame, CA) treated glass slides.

Deparaffinized and rehydrated sections were immunostained for the human PRL-R using the avidin-biotin immunoperoxidase method. The sections were pretreated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity and with 1.5% normal horse serum for 20 min to block any nonspecific binding sites. For immunostaining, the sections were sequentially exposed to the following solutions at 25 C: Appropriately diluted monoclonal antibody U5 [directed against epitopes to the extracellular domain of the rat PRL-R and does not interfere with the binding of PRL to its receptor (16)] which has been demonstrated to react well to the human PRL-R (16,21) or control mouse IgG (Cappel, Organon Teknika, Durham, NC). The concentration of antibody to achieve the best staining for each tissue was predetermined (25µg/ml for fetal membranes/decidua, 35µg/ml for placenta). The primary biotinylated antibodies were incubated with the sections at 4 C for 16-22 h, followed by the ABC reagent for 45 min. After each step, the sections were thoroughly rinsed in PBS (0.015mol/L, 3 changes, 3 min each). Peroxidase activity was demonstrated by incubation with a peroxidase substrate containing diaminobenzidine (DAB) 0.5 mg/ml with 0.01% hydrogen peroxide in Tris buffer (0.1mol/L, pH 7.2). The sections were rinsed in water, counterstained with hematoxylin, serially dehydrated and mounted with Pro-Texx (Baxter Diagnostics Inc., Deerfield, IL) and the slides were examined under bright field microscopy.

SDS polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Placentas with attached fetal membranes were obtained from women undergoing elective Cesarean section at term prior to labor at Kapiolani Medical Center for Women and Children

as described above. For preparation of tissue lysates intact or separated amnion, chorion and decidua (8g, 3g, 5g and 3g respectively) were rinsed three times in PBS, cut into small pieces and homogenized on ice for 20 sec at 2 min intervals for a total of 60 sec with a polytron homogenizer at a speed set at number 5 in buffer A, containing 20 mmol/L Hepes (pH 7.4), 32 mmol/L sucrose, 10 mmol/L EDTA, 1.0 mmol/L Phenylmethylsulfonylfluoride (PMSF), 0.18 mg/ml N-ethyl maleimide, 0.18mg/ml iodoacetamide and 0.38 mg/ml ovomucoid trypsin inhibitor), 3X vol of buffer to tissue. Homogenates were centrifuged at 14,000 g at 4 C for 5 min in a Sorvall RC-5B superspeed centrifuge (Dupont Company, Wilmington, DE), several aliquots of the supernatant (total lysate) were stored at -80 C until use; the remaining supernatant was centrifuged at 100,000g at 4 C for 60 min using a Beckman L3-40 ultracentrifuge. This supernatant (cytosol) was stored at -80 C until use and the resulting pellet was rinsed, resuspended in 2 mls buffer B [20 mmol/L hepes (pH 7.4), 32 mmol/Lsucrose, 0.5 mmol/LPMSF, 1.0 mmol/L EDTA, 2µg/ml aprotinin and leupeptin] and stored at -80 C until use. The protein concentration of the lysates was determined by the method of Bradford (22) using BSA as the standard and reagents purchased from Bio-Rad Laboratories, Hercules, CA.

For the intact and separated fetal membranes and decidual explants, the membranes were collected and dissected as described above, cut into 3 x 3 cm square pieces using a punch cutter and suspended in serum-free Dulbecco modified eagles medium (DMEM) (Gibco BRL, Grand Island, NY). A single explant was placed into a 60 x 10mm tissue culture dish in 5 ml of DMEM for 48 h under 5%CO₂-95% air at 37 C. Media was collected, concentrated eight-fold and subsequently washed to remove excess salt by centrifugation using Centraprep 10

(Amicon Inc., Beverly, MA) with a molecular weight cut off of 10,000 Daltons and stored at -80 C until use. Samples of amniotic fluid were obtained from women undergoing routine amniocentesis for chromosomal karyotyping and stored at -80 C until use.

Lysates (20µg protein), amniotic fluid samples (20 and 40 µg protein), and concentrated media (50µg protein) were electrophoresed in 10% SDS polyacrylamide under reducing conditions, and transferred to a polyvinylidene fluoride membrane (Immobilon, Millipore, Bedford, MA). The filters were preincubated at 37C for 1h in TBS-T (Tris-HCl, pH 7.6, 200mM NaCl and 0.1% Tween-20) containing 1% BSA (Sigma Chemical Company, St. Louis, MO) on an orbital shaker. The blots were then exposed to the primary antibody dissolved in the same buffer [U5 anti-PRL-R, 50µg/ml (16)] for 120 min. The blots were washed one time for 15 min and three times for 5 min in TBS-T, followed by horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham, Arlington Heights, IL) diluted 1:7500 in TBS-T containing 1% BSA for 1h, followed by two times 10 min and 2 times 5 min washes in TBS-T. The blots were then incubated with ECL Chemiluminescence substrate mixture according to the manufacturer's instructions (Amersham, Arlington Heights, IL) for 1 min and exposed to Fuji x-ray film (Fuji Photo film, Kanagawa, Japan) for 30 sec to 3 min.

D.Results

Northern blot analysis and quantitation

Autoradiograms of Northern blots hybridized under moderately stringent conditions with the ³²P labeled PRL-R cDNA probe showed strong hybridization signals in poly (A)⁺ RNA

samples from chorio-decidua and trophoblast tissues (Fig 2) lanes 1 and 2 respectively and a weaker but detectable signal in amniotic tissue (data not shown). The hybridization bands corresponded to three major mRNA species, 10.5 kb, 3.5 kb and 2.8 kb for all tissues Fig 2. An additional 7.3kb hybridization band was sometimes detected (not shown); when present it also varied in intensity in the tissues examined.

In order to determine if the stage of pregnancy and/or incidence of labor had any effect on the sum of the 2.8, 3.5 and 10.5 kb PRL-R transcripts, these were quantitated together in mRNAs from placental trophoblast and chorion plus adhering decidua collected at preterm delivery, (group 1), elective Cesarean section at term before labor, (group 2), Cesarean section during labor,(group 3), and after normal spontaneous labor/delivery, (group 4). The PRL-R transcripts (10.5, 3.5 and 2.8 kb) were quantitated by expressing them as a percentage of G3PDH. Since the 7.3 kb transcript was often absent, it was omitted from the quantitation. The means and standard errors (SE) for the PRL-R transcripts expressed in the trophoblast and chorio-decidua increased significantly ($P<0.05$) after labor and delivery (group 4) when compared to the tissues collected before labor (group 2) Fig 3A and B. This increase noted in tissue obtained after labor and delivery occurred late in labor since the levels in both tissues were low during labor (group 3). The greater variation at this time (group 3) in both tissues, suggests the expression of the PRL-R is changing over this period. In contrast, there was no significant difference between any of the same four groups of patients in the levels of PRL mRNA expressed in the same chorio-decidua mRNA preparations using the same filter after deprobing, washing and rehybridization (Fig 3C). The PRL mRNA levels showed particularly large standard errors in the group 2 and 4 patients not seen in the PRL-R mRNA in these

samples. These different patterns of expression of the PRL and PRL-R genes in the same tissues suggest that the actions of PRL is modulated separately by control of PRL and PRL-R levels.

The PRL-R transcripts hybridizing at 10.5, 3.8 and 2.5 kb were quantitated separately in poly (A)⁺ RNA from each tissue at the same time point; placental trophoblast (n=2), amnion (n=8), chorion (n =2) and decidua (n=5), all collected before labor at term elective Cesarean section, (Fig 4A). The results are expressed as a percentage of the total transcripts expressed for each tissue. The major transcript in all tissues was 10.5kb which was expressed in similar amounts in all tissues. The 3.5 and 2.8kb transcripts were expressed at lower levels but in approximately equal proportions in each of the tissues studied (Fig 4A).

PRL-R transcripts were quantitated separately in the trophoblast and chorio-decidua in the peripartal period, in tissues collected at different time points. The 3.8 and 2.5 kb transcripts ran too close together to be quantitated separately and were therefore pooled. The 10.5 kb transcript showed the greatest change in trophoblast and chorio-decidua obtained after labor and delivery (group 4). In the trophoblast (Fig 4B) this transcript showed a marginal but not significant decline during labor (Fig 4B, group 3) not reflected in the chorio-decidua of the same patients, (Fig 4C, group 3). The expression of the 10.5 kb transcript was greater than the combined 3.5 and 2.8 kb transcripts at all time points in chorio-decidua, unlike the trophoblast (Fig 4C), which showed less of the 10.5 kb transcript at preterm delivery (group 1), and during spontaneous term labor (group 3).

In Situ hybridization

Sections of placental trophoblast and fetal membranes with adhering decidua from preterm delivery (n=5), and term tissues, collected both before (n=4) and after (n=4) spontaneous labor and delivery, were hybridized with oligo probes directed to an area of the extracellular and intracellular domain of the PRL-R. No differences in the hybridization patterns were seen in amnion, chorion and decidua tissue obtained at preterm and term. After 14 days exposure, a strong hybridization signal was localized to both the chorionic cytotrophoblast cells and the decidua, seen in the darkfield (Fig 5B) photomicrograph but not visible in brightfield (Fig. 5A) at this level of gene expression. The signal in these layers was not always continuous as shown in the chorionic cytotrophoblasts (Fig 5A and B), and often varied in intensity in different areas in a single section. There was no detectable signal in the amnion or amniotic epithelium with this technique. Although the amniotic epithelium is absent from the section in Fig 5A and B, it is seen partially detached from the chorion in Fig 5C in brightfield and Fig 5D darkfield. The control sense probes gave no signal as shown in Fig 5E and F (bright and darkfield views respectively), an increased non-specific signal can also be seen on the glass and not associated with the tissue in this control (Fig.5F).

The strongest hybridization signal in the placenta was seen after 14 days exposure over the syncytiotrophoblast of the placental villi, a low power brightfield photomicrograph is shown in Fig 6A with its corresponding darkfield view Fig 6B. Although identical hybridization patterns for both preterm and term tissues were obtained however, the intensity of the signal over the syncytiotrophoblast at preterm was generally brighter. Signals in the preterm and

term tissues were intermittent with areas of intense, moderate and low hybridization in a single view. A high power brightfield view of intense hybridization is shown in Fig 6C and in darkfield Fig 6D. The complete lack of hybridization signal in the placenta using the control sense oligo probes is shown in the brightfield and darkfield views (Fig 5E and F respectively).

Immunocytochemistry

The PRL-R was localized by immunocytochemistry in the amnion, chorion, decidua and placenta. In all sections from preterm (n=5) and term tissues, collected both before (n=5) and after (n=5) spontaneous labor and delivery there was strong continuous cytoplasmic staining in the amniotic epithelial cells (Fig. 7A). However, at high power (Fig 7C), the cells were not uniformly stained, with some cells showing little or no stain and others showing a strong stain particularly dense in the apical region. There was also variability in the intensity of the cytoplasmic stain in the cells of the chorionic cytotrophoblast (Fig7A), with more intense staining in the cytotrophoblast cells located closest to the decidua. These results suggest that translation is not synchronized on a tissue-wide scale. The decidual cells on the other hand showed lighter and much more uniform cytoplasmic staining (Fig 7A). At high power the staining of the chorionic cytotrophoblast appeared localized in some cells to their periphery (Fig 7D), possibly indicating a pericellular localization of the PRL-R. When IgG was used in place of the primary antibody on serial sections of fetal membranes and decidua, there was no staining, shown in Fig 7B. The immunostaining of the placental trophoblast was predominately in the cytoplasm of the syncytiotrophoblast lining the perimeter of the placental villi (Fig 7E), shown at a higher magnification in Fig 7F. The distribution and intensity of staining of the syncytiotrophoblast varied considerably between tissues in the same group of patients and

was 'patchy' in all sections similar to the *in situ* hybridization patterns obtained. The adjacent basal plate region had some cells staining heavily at equal intensity to that of the syncytiotrophoblast, other cells however, remained unstained, (Fig 7E). The amount of antibody required for the optimal staining of the placental sections was greater than that required for the fetal membranes, suggesting that there is less of the translated PRL-R protein in the placenta than in the fetal membranes and decidua. There was no qualitative difference in the amount or distribution of staining in the preterm and term tissues, however, only 3 of the 5 placentas obtained at Cesarean section prior to labor showed any staining, whereas 5 of 5 stained positively in those collected after spontaneous labor and delivery.

Western blot analysis of decidua, fetal membranes and amniotic fluid

Separated amnion, chorion and decidual tissues were used for the preparation of cytosol fractions or incubated as explants for 48h and the media concentrated and analyzed by Western blotting. The results are summarized semi-quantitatively for clarity in Table 1 and the experimental data shown in Fig 8. Six major molecular species of 95, 85, 63, <63, >30 and 30 kDa were detected by the antibody (U5), directed to the extracellular domain of the PRL-R (16, 17, 23), in the cytosol from separated amnion, chorion and decidua (Table 1). The two bands at 95 and 85 kDa were difficult to size accurately from their positions in these gels and are therefore designated as between 95-85 kDa (Table 1 and Fig. 8). Both bands could be readily identified in the cytosol from decidua and chorion and probably represent mature glycosylated forms of the human PRL-R, Fig 8A, lanes 3 and 4 respectively. They were also detected but with less intensity in the media after incubation of amnion, chorion and decidua as intact explants from one patient (Fig. 8A, lane 1), but not in that from a second patient (Fig 8B,

lane 5). They were absent from the media after incubation of separated amnion, chorion and decidua (Table 1, Fig 8B, lanes 2, 3 and 4 respectively). Additional PRL-R related proteins indicate the partial degradation of the PRL-R in spite of the addition of protease inhibitors to buffers used in all procedures. This is similar to previous reports for the rabbit PRL-R in the mammary gland (23).

Thus 63 and 30 kDa PRL-R related species were detected in both the cytosol and media from amnion, chorion and decidua and were also present in amniotic fluid (Table 1, Fig 8B, lanes 2-4). The intensity of these bands varied from patient to patient as seen in the media after incubation of amnion, chorion and decidua from two patients both after elective Cesarean section at term before labor, Fig 8A lane 1, Fig 8B lane 5. The variability could reflect the active changes occurring in the prepartum period and/or reflect differences in the levels of proteases in their tissues or minor differences in the time of storage of these samples at -80C for up to two weeks. The <63 kDa species detected in cytosol and media from amnion, chorion and decidua Fig 8B, lanes 2-4 was absent from amniotic fluid as was the >30 kDa species most readily detected in the cytosol and media from the decidua (Fig 8A, lane 3 and 4 respectively). This was however a minor species detected in the cytosol and media from the chorion (Table 1, Fig 8B, lane 4) and was absent from either in the amnion (Table 1, Fig 8B, lane 2). No protein bands were detected in control media (media concentrated after 48 h incubation without any tissue), Fig 8A, lane 2, Fig 8B lane 1. Likewise no protein bands were detected in media of cytosol when the primary antibody was omitted from the Western blot protocol (data not shown).

E. Discussion

A comprehensive examination of the transcription and translation of the PRL-R gene in human intrauterine tissues in the peripartal period has been carried out using a number of complementary techniques. In situ hybridization histochemistry and Northern analysis have been used to localize and quantitate the expression of the PRL-R gene respectively. Immunocytochemistry has been used to localize translated proteins, while Western blot analysis allowed us to study the intracellular and extracellular distribution of receptor and receptor related proteins.

The results from this range of techniques for both gene and protein are in remarkably good agreement and show that the PRL-R is widely expressed by both fetal and maternal tissues in late pregnancy. The chorionic cytotrophoblast of the chorion laeve were shown to transcribe and translate this receptor at relatively high levels by both Northern analysis and immunocytochemical localization, in agreement with studies reported using particulate membrane preparations from these tissues (9,10). In situ hybridization histochemistry verified its transcription by the chorionic cytotrophoblast cells, but this technique gave a lower intensity signal in the decidua and amnion than would be expected from the immunolocalization studies, which showed the amniotic epithelium intensely stained and the decidua cells staining well but somewhat less strongly than the chorionic cytotrophoblast. Nothing is known about the half-life of the human PRL-R mRNA in these tissues or the turnover of the receptor itself, but the data from the in situ hybridization might suggest discontinuous transcription in the chorion and decidua. The strong immunolocalization to the amniotic epithelium in the absence of an in situ

hybridization signal also suggests quite disparate levels of gene expression and protein turn over. Northern analyses with pooled amnion showed that this fetal layer does indeed produce the PRL-R and is not merely a site of sequestration of a possible PRL-BP complex. Controversy whether PRL itself is a product of these cells (24), probably arose because immunolocalization does not readily distinguish between hormone produced and that bound to a receptor or binding protein. Thus, our demonstration that some amniotic epithelial cells lack the receptor protein are in agreement with the immunolocalization studies previously carried out with antisera to PRL (2,25).

The decidua was the first extrapituitary site recognized as producing PRL (1,26,27), and a paracrine role for this hormone was recognized early because the high levels in amniotic fluid were not in equilibrium with those of the maternal circulation (27). For several years immunolocalization studies of PRL with antisera to PRL misled the field into the belief that the placenta was also a site of PRL production (23,28,29). This is not surprising in view of the sequence homology between PRL and placental lactogen, which is present in placental syncytiotrophoblast in very high concentrations (30,31). Thus molecular techniques were essential to show unequivocally that the placental syncytiotrophoblast is not a site of PRL production. Although the data presented here show for the first time that the placental syncytiotrophoblast transcribes and translates the PRL-R, albeit at lower levels of expression than the chorion and decidua. The source(s) of the ligand for this receptor may be multiple, the high levels of PRL in amniotic fluid derived principally from the decidua (1,4,26) suggest that this is the major source of ligand capable of initiating an as yet unknown biological function(s)

in the placenta. Another and more directly available local source of PRL is the placental basal plate (*decidual basalis*) (2).

The expression of the PRL-R in the decidua itself was shown by Northern blot analysis and by *in situ* hybridization. Immunolocalization showed a light but uniform cytoplasmic staining in the decidual cells. Together, this implies an autocrine/paracrine role for PRL in the decidua. The heterogeneous nature of this tissue with 47% bone marrow derived cells at term and a relatively new but well defined role for PRL as an immunocrine hormone (32) may reflect a function in the activation of this tissue at term (33).

Quantitative Northern analysis showed a 10.5 kb PRL-R transcript as the major transcriptional product in all intrauterine tissues, in addition to confirming the presence of the 3.5 and 2.8 kb transcripts (12,34) . The 10.5 kb PRL-R transcript has been described previously in breast (11) and appeared to be the most significant transcript in uterine tissues since it was modulated with respect to the time of labor and delivery. We have used the rehybridization of the same Northern filters to show that the transcription of PRL and its receptor in these tissues are not temporally linked: an *a priori* need perhaps for a PRL binding protein as a buffer and store. The increased transcription of the PRL-R in both the chorio-decidua and placental trophoblast commencing during labor was unexpected and would be too late for the proposed osmoregulatory functions of decidual PRL (15). We have shown that PRL added to human amnion and chorion cells *in vitro* caused a release of collagenolytic activity into the medium of these cells (35). Further work in this laboratory indicates that PRL is one of the local hormones at the maternal-fetal interface capable of increasing the transcription and translation of specific metalloproteinases in late pregnancy, while an

osmoregulatory role earlier in gestation is not precluded. The sequential orchestration of specific enzymes to degrade components of the extracellular matrix of the fetal membranes and placenta in the last weeks of pregnancy is central to our understanding of peripartal events and human parturition (36).

The Western blot data using cytosol from separated amnion, chorion, decidua and media from intact explants of these tissues show the mature glycosylated forms of the PRL-R as two bands at approximately 95-85 kDa, which represent differing degrees of glycosylation of this receptor. We have used an immunoblot method considerable less sensitive than the detection system used by Okamura et. al.,(23) and relatively crude heterogeneous preparations in the forms of cytosol and media after incubation of these cells it is not therefore surprising that we detected multiple immunologically related PRL-R products. Indeed, multiple bands were also detected using the same U5 antibody for the PRL-R in the rat ovary and prostate and the pig and rabbit mammary glands (23). These represent proteolytic degradation products derived from the PRL-R, in spite of the addition of protease inhibitors and the storage of samples at -80C. However, it is not possible to distinguish between degradation products and specific proteolytic cleavage which could give rise to shortened potentially functional shorter forms of the PRL-R, which may be binding and/or transport protein(s) released from the cells to carry the PRL ligand across the decidual cells and their basement membrane and/or the extracellular matrix of the amnion and chorion and hence into amniotic fluid. Further work is required to demonstrate a relationship between the 30 kDa PRL-R product described here and a PRL binding protein recently described in human milk (14). This species as well as the 63 kDa protein were detected in the cytosol and media from the amnion, chorion and decidua as well

as in amniotic fluid. Their presence in amniotic fluid suggests their release into this medium in vivo and lends credence to the possibility of them having some functional significance in these tissues. The decidua was unique in processing an additional >30 kDa molecular weight species detected in high amounts in both cytosol and media from these cells. However, it was undetectable in the amnion and in amniotic fluid. The use of antibodies to known epitopes of the human PRL-R would help to clarify the structural relationships of these PRL-R products and provide some further insight into their potential function(s) in these tissues.

In summary, our data show that the autocrine/paracrine systems for PRL/PRL-R at the maternal-fetal interface of the fetal membranes are more complex than originally thought. The transcription and translation of the PRL-R gene in the human chorionic cytotrophoblast in decidua, in amnion, in as well as in the placental syncytiotrophoblast has been shown at times in pregnancy where tissues are accessible. Of the six molecular species detected by Western analysis, two, 95-85 kDa, appear to be the classical forms of the glycosylated receptor protein. These are proteolytically cleaved in a tissue specific manner and two (63 and 30 kDa species) were present in amnion, chorion and decidua as well as in amniotic fluid. Further work is required to clarify whether these proteins derived by cell-specific processing of the translated product of the single PRL-R gene may function as PRL binding/transport proteins.

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FIG. 1. Schematic representation (not drawn to scale) of the full length PRL-R cDNA, the extracellular domain, transmembrane (TM) and intracellular domains. *PST I* digestion of the full length cDNA released the 1350 bp cDNA probe (hatched box). The nucleotide sequences of the 48mer probes derived from the extracellular and intracellular domains are given, the arrows indicate that these were synthesized in both the sense and antisense directions.

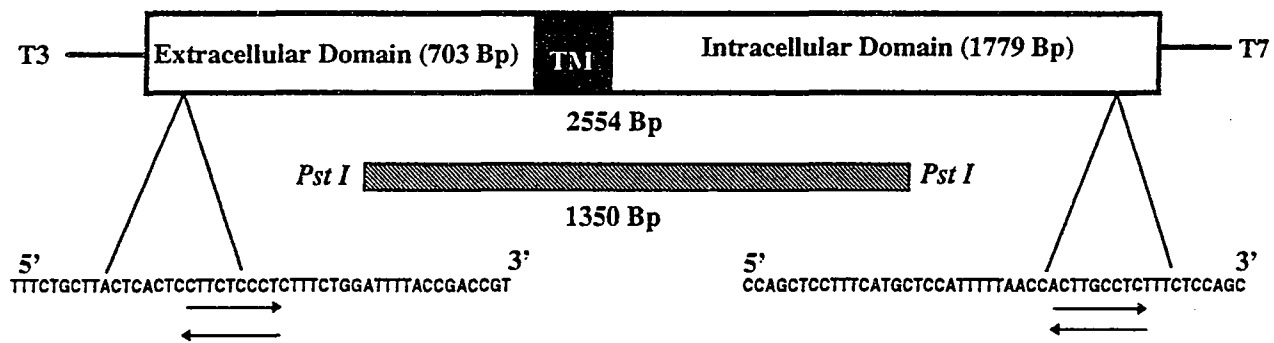


FIG 2. Northern blot analysis of poly(A)⁺ RNA (20μg) from chorio-decidua (lane 1) and placental trophoblast (lane 2) hybridized and washed under moderate conditions. The autoradiograph was exposed for 16h. kb, kilobase.

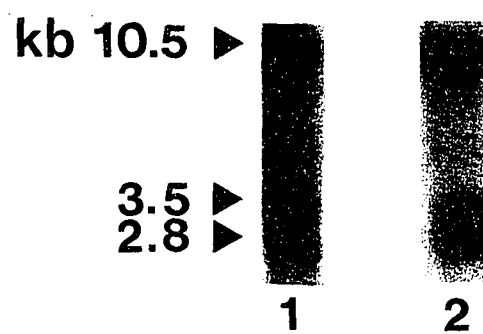


FIG 3. Quantitation of total (10.5+3.5+2.8 kb) PRL-R mRNA transcripts (the 7.3 transcript was omitted from this quantitation), in human intrauterine tissues obtained from four groups of patients (1) preterm labor and delivery (n=6), (2) elective term Cesarean section (n=18), (3) emergency Cesarean section during labor (n=5) (4) after normal labor and delivery (n=17). The positive signals from the autoradiograms were quantitated against the expression of G3PDH in the same sample. Panel A, placental trophoblast; Panel B, chorio-decidua and Panel C, the same filter as in Panel B, deprobed and rehybridized with the PRL cDNA probe and quantitated against the expression of G3PDH. Mean \pm SE,* significantly different from each other (p<0.05).

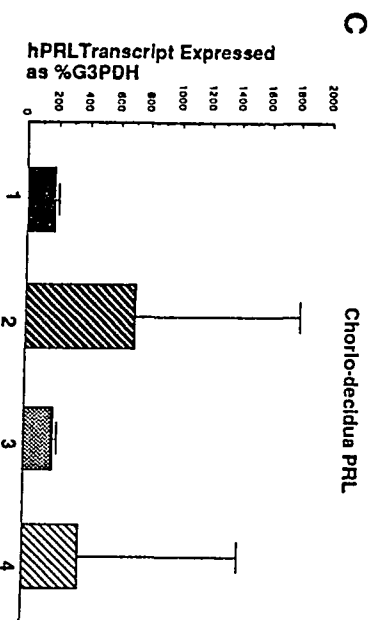
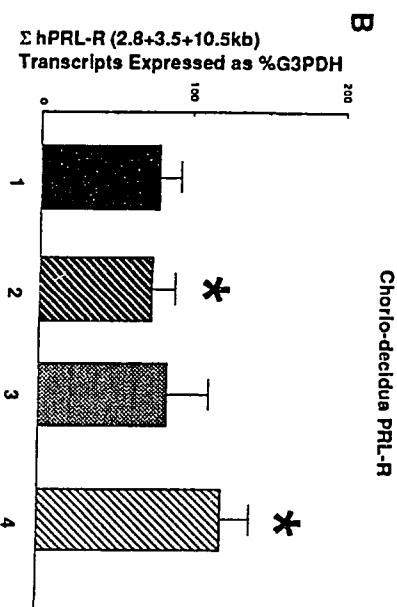
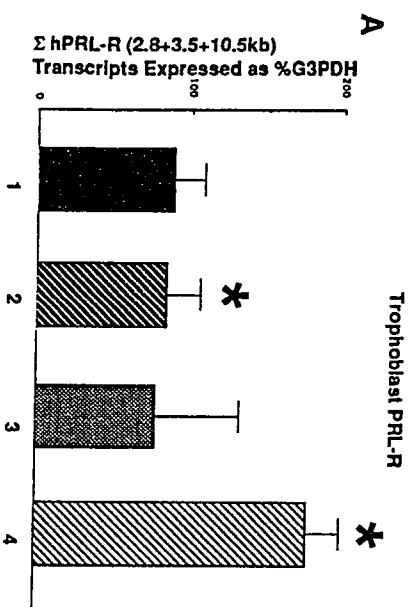


FIG 4. Panel A; quantitation of the individual PRL-R transcripts (10.5, 3.5 and 2.8 kb) in poly(A)⁺ RNA from pooled intrauterine tissues collected at elective term Cesarean section before labor. Each transcript was expressed as a percentage of the total transcripts (10.5, 3.8 and 2.8 kb) expressed in that tissue. Panel B; quantitation of the 10.5 and the pooled 3.5 + 2.8 kb transcripts expressed as a percentage of G3PDH in the placental trophoblast (Panel B) and chorio-decidua (Panel C) in tissues collected during the peripartal period (same patients as in Fig 3), (1) preterm labor and delivery (n=6), (2) elective term Cesarean section (n=18), (3) emergency Cesarean section during labor (n=5) and (4) after normal labor and delivery (n=17).

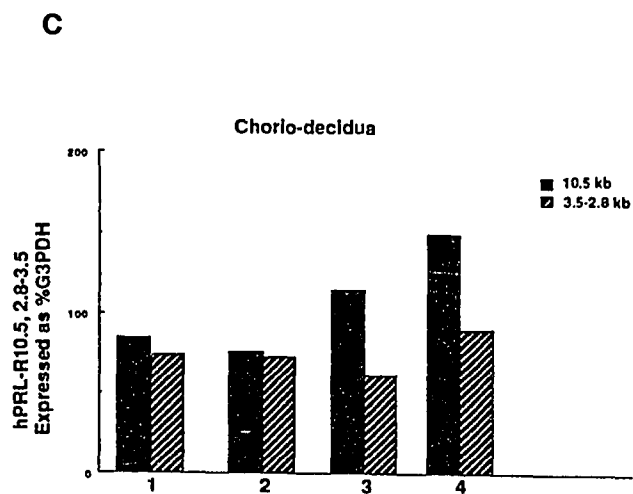
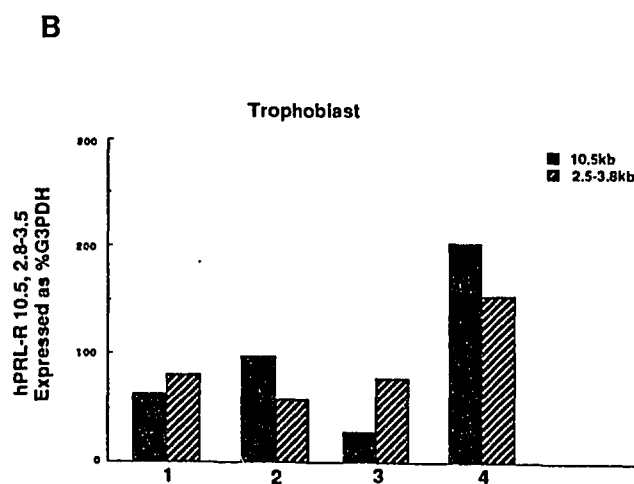
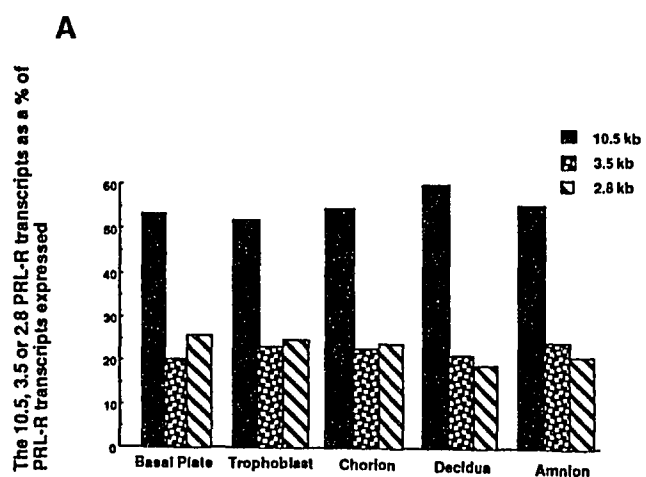


FIG 5. Localization of the PRL-R mRNA by in situ hybridization in amnion, chorion and decidua from the human placenta of term pregnancy collected prior to labor, after 14 days exposure. A and B, bright and darkfield views after hybridization with the antisense oligo probes, showing the signal in the chorionic cytotrophoblast (c) and the deeper layers of the decidua (d). The signal in the cytotrophoblast is shown especially bright in one area (B) which disappears, although the cytotrophoblast layer continues (A), X130. There was no signal in the amnion after a 14 day exposure, although the chorionic signal was bright, shown in brightfield (C) and darkfield (D), X100. A representative section hybridized with sense 48mer probes and processed identically was negative, as shown in both bright and darkfield views respectively (E and F), X120.

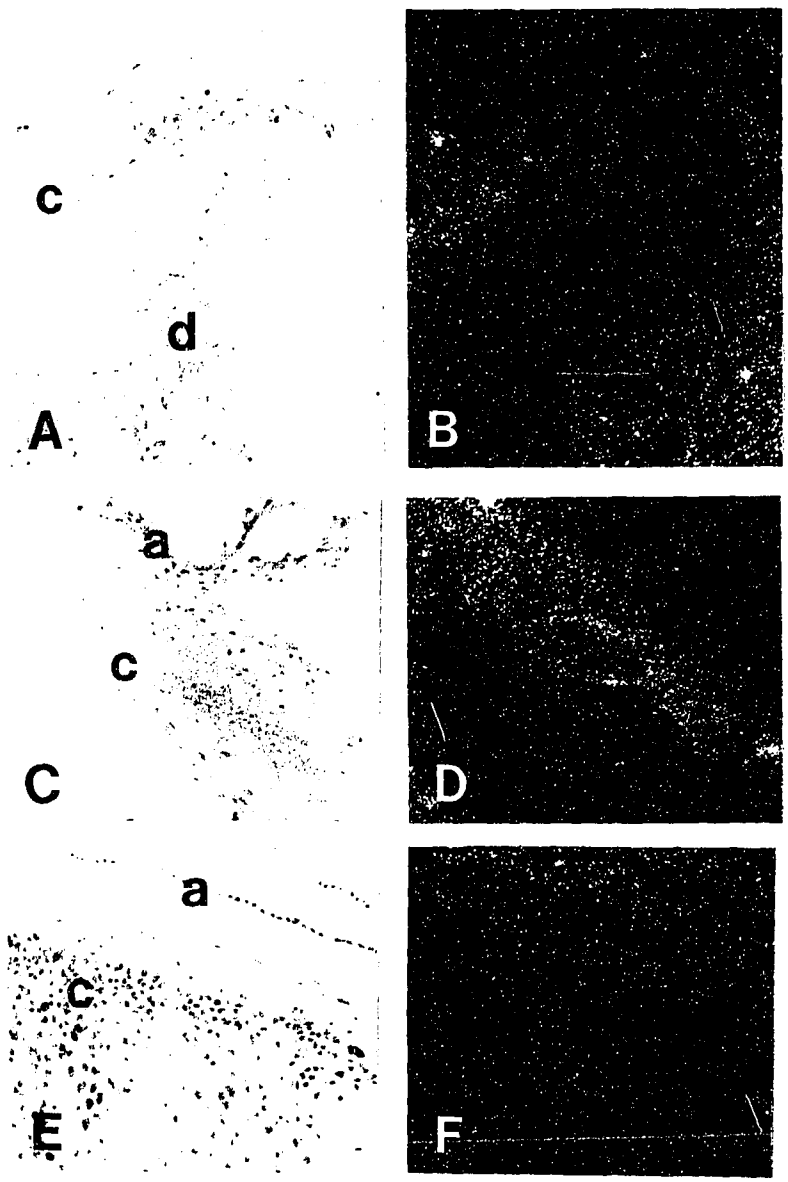


FIG 6. Localization of the PRL-R mRNA by in situ hybridization in the human placenta of term pregnancy collected prior to labor, after 14 days exposure. The signal was intermittent with areas of intense, moderate and low hybridization in the syncytiotrophoblast(s), shown in bright and darkfield views, A and B, X160. An area of intense hybridization in the syncytiotrophoblast is shown in bright and darkfield (C and D) at higher magnification, X400. A section hybridized with the sense 48mer probes and otherwise processed identically was negative and shown in both bright and darkfield views respectively (E and F), X150.

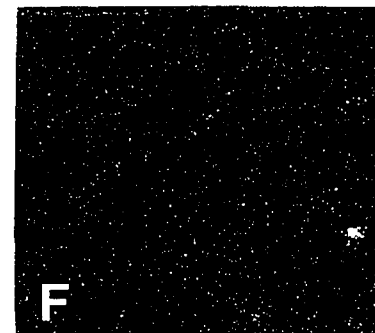
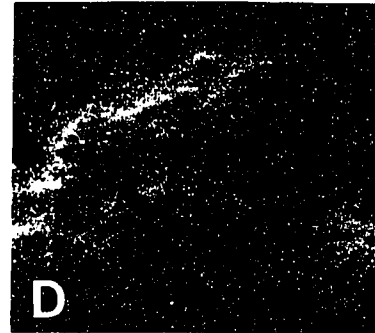
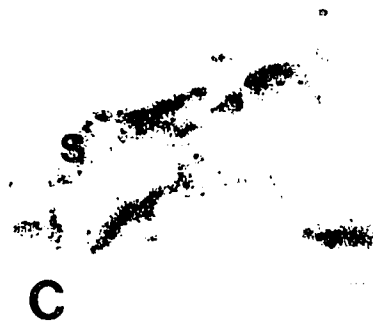
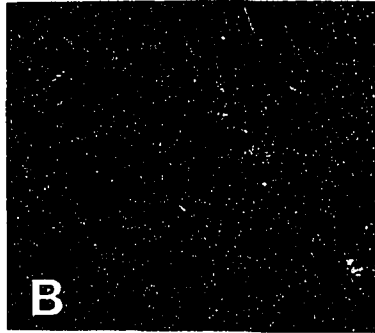


FIG 7. Immunolocalization of the PRL-R in the human fetal membranes, decidua and placenta collected at term Cesarean section prior to labor. A Mab to an epitope of the extracellular domain of the rat PRL-R (U5) was used at 25 $\mu\text{g}/\mu\text{l}$ for the fetal membranes and 35 $\mu\text{g}/\mu\text{l}$ for the placenta. Strong cytoplasmic staining was obtained in the amniotic epithelium (a) shown at low power (X190) in A and high power (X670) in C. At high power, some cells lacked stain (open arrow) and others showed intense staining particularly in the apical region (closed arrow). Variability of the cytoplasmic stain in the cells of the chorionic cytotrophoblast (c) with those situated closer to the adhering decidua (d) staining most intensely (A). At higher power (X640) the stain in some cells appeared most intense around their periphery (D). The decidual cells on the other hand, showed lighter but uniform cytoplasmic stain (A). A serial section treated identically except for use of mouse IgG at the same concentration as the primary antibody, showed complete lack of stain (B), X190. The immunolocalization of the PRL-R in the placenta is shown in the syncytiotrophoblast of the villous trophoblast (E). In addition, some cells stained well while others remained unstained in the placental basal plate (bp), X170. The cytoplasmic stain in the syncytium is shown at high power (F), X610.

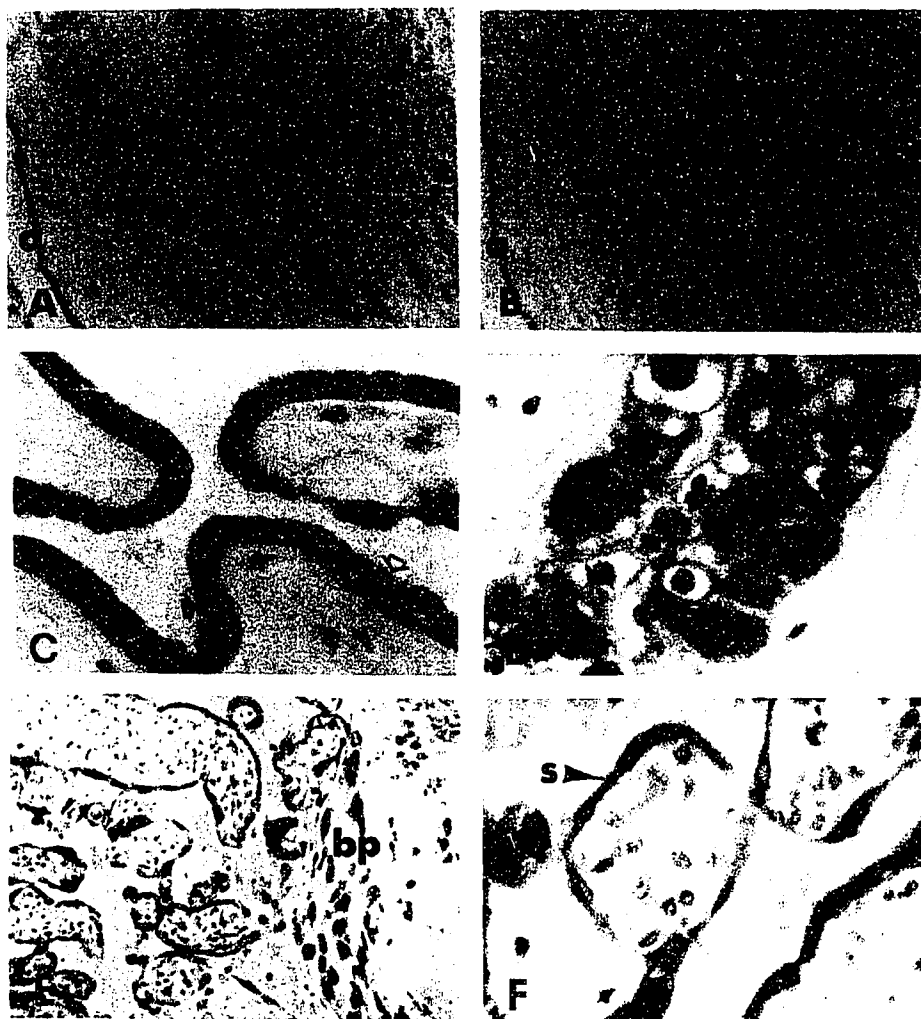
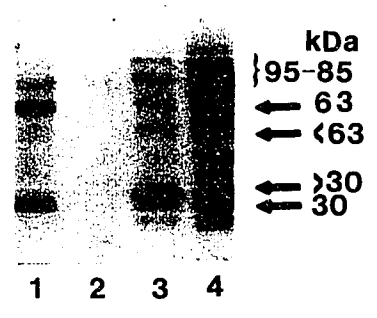


Fig 8. Western blot analysis of the PRL-R using the antibody to the rat PRL-R (U5). The mature glycosylated forms of the PRL-R were detected as two bands at approximately 95 and 85 kDa in the cytosol from decidual cells (A, lane 3) and chorion cells (A, lane 4). These were also present but less intense in the media from amnion, chorion and decidual explants, when these were kept intact, shown in one patient (A, lane 1) but absent from media from a second patient (B, lane 5). Multiple proteolytic degradation products were detected in both cytosol and media from amnion, chorion and decidua; the major products of 63 and 30 kDa were detected in differing amounts in the cytosol of amnion (not shown), chorion (A, lane 4) and decidua (A, lane 3). These were also present in amniotic fluid (not shown). However, the major product in the cytosol of decidual cells was a protein of >30 kDa (A, lane 3) also present in the media from these cells after incubation (B, lane 4). media from the separated amnion, chorion and decidua also showed a product of <63 kDa (B, lane 2, 3 and 4 respectively). No protein bands were detected in concentrated media incubated for 48 h in the absence of tissue (A, lane 2, B, lane 1).

A



B

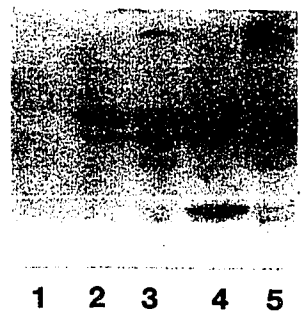


Table 1. Summary of Western blot analysis using cytosol obtained from elective Cesarean tissue, separated amnion, chorion and decidua and in concentrated media obtained from these separated layers incubated for 48h in vitro. For comparison Western blot analysis of amniotic fluid is shown.

kDa	Decidua		Chorion		Amnion		Amniotic Fluid
	cytosol	media	cytosol	media	cytosol	media	
95-85	+	ND	++	ND	+	ND	ND
63	++++	+++	++++	++++	++	++	++++
<63	+++	+++	+++	++++	++	++	ND
>30	++++++	++++	++	++	ND	ND	ND
30	++	+++	+++	+++	++	++	+++

CHAPTER III
CHARACTERISTICS OF THE BINDING OF ³²P-LABELLED HUMAN
RELAXINS TO THE HUMAN FETAL MEMBRANES.

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A. Abstract

The two human relaxin genes termed H1 and H2 are expressed in the choriodecidua and placenta and have been proposed to act via specific receptors as local modulators of collagenolysis in the fetal membranes. Such receptors have been inferred, but not demonstrated, from studies of the effect of adding exogenous relaxin to these tissues. Thus conditions were optimized for the binding of ^{32}P -labelled human relaxin H2 to membrane-enriched particulate fractions of human fetal membranes, amnion and chorion, with adhering decidua. The membrane protein concentration was optimal at 250 μg , when incubated at 27 $^{\circ}\text{C}$ for 60 min, at pH 7.5 with Mn^{2+} and Mg^{2+} ion concentrations of 2.0 mM. Incubation of membrane particulate fractions with increasing amounts of labelled relaxin H2 suggested the presence of a single class of binding sites with an affinity constant (K_d) of 2.15 nM. The binding was primarily to the chorion and decidua with very little to the amnion layer. The competition for binding of the ^{32}P -labelled human relaxin H2 with unlabelled relaxin H2 gave an IC_{50} of 28 pM, while an IC_{50} of 60 pM and 280 pM was obtained for relaxin H1 and porcine relaxin, respectively. In contrast unlabelled guinea pig relaxin inhibited this binding by only 10 % even at a 1000-fold greater concentration than H2, and human recombinant insulin failed to inhibit even at a million-fold concentration of unlabelled relaxin H2.

Relaxins H2 and H1 can readily displace the binding of either ^{32}P -labelled human relaxins H1 or H2 and gave very similar displacement curves. The binding affinity of relaxin H2 however, was 5-fold higher than that of relaxin H1. These data provide evidence for the presence of a relaxin receptor that may preferentially bind relaxin H2.

B. Introduction

Relaxin is a polypeptide hormone belonging to the insulin super family and is best known for its role in parturition (Sherwood, 1994; Bryant-Greenwood & Schwabe, 1994). Two human relaxin genes termed H1 and H2 have been identified, the H1 gene from a genomic DNA library (Hudson *et al.* 1983) and the H2 gene from a corpus luteum cDNA library (Hudson *et al.* 1984). The corpus luteum of the cycle (Ivell *et al.* 1989) or pregnancy (Hudson *et al.* 1984) expresses only relaxin H2 at a high level since it is the sole source of circulating relaxin (Bryant-Greenwood & Schwabe, 1994). However, both genes are expressed, albeit in lower levels consistent with local autocrine/paracrine actions in the placenta, fetal membranes and decidua (Sakbun *et al.* 1990; Hansell *et al.* 1991), prostate (Ivell *et al.* 1989; Hansell *et al.* 1991), and breast (Tashima *et al.* 1994). The biological actions of relaxin are presumed to be mediated through specific receptors located on its target cells, however, the structure of the relaxin receptor in any species is currently unknown (Bryant-Greenwood & Schwabe, 1994). The identification and characterization of a relaxin receptor in a target tissue is an essential step towards its purification or identification of its gene. It is also important for substantiating relaxin's systemic and autocrine/paracrine role(s) (Bryant-Greenwood, 1991).

Porcine relaxin cannot be labelled directly with ¹²⁵-iodine since it contains no tyrosine residues (Sherwood & O'Byrne, 1974), however indirect methods have been developed and the product used for the characterization of relaxin binding to plasma membrane-enriched particulate fractions isolated from rat myometrium, a target for relaxin in late pregnancy (Mercado-Simmen *et al.* 1982). Relaxin is important in the rat in late pregnancy not only to effect uterine quiescence but also to facilitate cervical dilatation (Sherwood, 1994). Studies in the pig indicated a different hormonal control of the putative relaxin receptor in the uterus and cervix (Mercado-Simmen *et al.* 1982). The

same labelled porcine hormone was also used to show specific binding of relaxin to particulate preparations from human fetal membranes (Koay *et al.* 1986). Porcine relaxin added *in vitro* to cultured amnion/chorion cells increased the release of plasminogen activator and collagenolytic activity into the medium. In addition, an *in vivo* correlate of these *in vitro* results was obtained by detection of increased plasminogen activator in amniotic fluid in the period 32-40 weeks gestation (Koay *et al.* 1983).

Many laboratories experienced difficulties in demonstrating specific binding using ^{125}I -porcine relaxin as discussed in Greenwood *et al.* 1981. The central problem appeared to be the preparation of a monocomponent biologically active ligand. Two laboratories have devoted considerable effort to this particular problem; one produced a monocomponent high specific activity carrier free ^{125}I -porcine relaxin and used it to show specific ligand binding in mouse uterus, symphysis pubis, ovary and brain (Yang *et al.* 1992) and the other, developed a ^{32}P -labelled recombinant human relaxin H2 and used it to show displaceable binding on rat uterus, cervix, brain and heart atrium (Osheroff *et al.* 1990, 1992). The efforts of these laboratories encouraged the reactivation of work on the human fetal membranes using homologous human labels. The recent novel chemical synthesis of human relaxins (Bullesbach & Schwabe, 1991) has made it possible to use both synthetic human relaxins H1, H2 to further characterize their binding in the human fetal membranes and decidua.

C. Materials and Methods

Materials

Synthetic human relaxins H1, H2 were chemically synthesized by solid-phase peptide synthesis in the laboratory of Drs. C. Schwabe and E. Bullesbach, Medical University of South Carolina. Guinea pig relaxin was similarly prepared with its structure based upon

the published cDNA sequence (Lee *et al.* 1992). Porcine relaxin (CMA') was isolated in our laboratory from pig ovaries using the method of Sherwood & O'Byrne , (1974). Human recombinant insulin was obtained from Intergen Company, Purchase, New York, U. S. A.

Particulate membrane preparation.

Placentas with the fetal membranes attached were obtained from women undergoing elective Cesarean section at term prior to labor at Kapiolani Medical Center for Women and Children (Honolulu, Hawaii) with Institutional Review Board approval and approval of the Human Experimentation Committee of the University of Hawaii. Fetal membranes (amnion, chorion plus adhering decidua) were immediately cut free from the placental disc around its periphery, suspended in ice cold phosphate buffered saline (PBS) and transported on ice within 1 h of removal. Membrane-enriched particulate fractions were prepared as described by Mercado-Simmen *et al.* (1980) with modifications. Tissues (20 g) were rinsed three times in PBS, suspended in 9 ml homogenization buffer A (20 mM Hepes (pH 7.4), 32 mM sucrose, 10 mM EDTA, 1.0 mM phenylmethylsulfonylfluoride (PMSF), 0.18 mg/ml N-ethyl maleimide, 0.18 mg/ml iodoacetamide and 0.38 mg/ml ovomucoid trypsin inhibitor) per gram wet weight, cut into small pieces and homogenized on ice three times for 20 sec at 2 min intervals with a Polytron homogenizer (Brinkmann, Westbury, N. Y.) at a speed set at number 5. Homogenates were centrifuged at 14,000 g at 4 °C for 5 min in SS34 rotor of a Sorvall high speed centrifuge (Dupont Company, Wilmington, Delaware, U. S. A.), the supernatant was collected and centrifuged at 100,000 g at 4 °C for 60 min in a type 60 Ti rotor using a Beckman preparative ultracentrifuge. The resulting pellet was rinsed and resuspended in 2 ml buffer B (20 mM Hepes (pH 7.4), 32 mM sucrose, 0.5 mM PMSF, 1.0 mM EDTA, 2 µg/ml of aprotinin and leupeptin) to remove most of the EDTA then recentrifuged in Beckman TL-100

ultracentrifuge at 200,000 g, 4 °C for 30 min. The pellet was collected, rinsed and resuspended in 2 ml buffer C (same as B without EDTA plus 20 mM MgCl₂) for the potential unmasking of the receptor (McWey *et al.* 1982). After incubation on ice for 60 min, the suspension was centrifuged as above, the pellet rinsed and resuspended in buffer D (20 mM Hepes (pH 7.4), 32 mM sucrose, 0.5 mM PMSF, 10 µg/ml of aprotinin and leupeptin, 50 mM NaCl and 0.1% (w/v) bovine serum albumin), which was also used as the buffer for the binding assays. To minimize possible degradation of membrane proteins the whole procedure was performed at 4 °C and protease inhibitors were added in all the buffers used (Mercado-Simmen *et al.* 1980). All the chemicals and reagents used for processing the tissues were obtained from Sigma Chemical Company, St. Louis, Missouri, U. S. A. The concentration of protein was determined after the method of Bradford (1976) with BSA as the standard using reagents purchased from Bio-Rad Laboratories, Hercules, California, U. S. A., and the final concentration of membrane proteins was adjusted with binding buffer D to 1 mg/ml.

Phosphorylation of human relaxins.

Human relaxins H1 or H2 were phosphorylated with g³²P-ATP (6,000 Ci/mmol; Dupont NEN Research Products, Boston, Massachusetts, U. S. A.) using the method as described by Osheroff *et al.* (1990) with minor modifications. A total reaction volume (30 µl) containing 10 µg relaxin H1 or H2, 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM MgCl₂, 100 µCi g³²P-ATP and 250 U of lyophilized catalytic subunit of cyclic AMP-dependent protein kinase (obtained from bovine heart muscle; Sigma Chemical Company, St. Louis, Missouri, U. S. A.) was used. The reaction mixture was incubated for 2 h at 27 °C, placed on ice to stop the reaction and loaded onto a Sep-Pak C18 cartridge (Waters Chromatography, Millipore Corporation, Milford, Massachusetts, U. S. A.). This

had been presaturated with 2% BSA, equilibrated and washed with 0.1% trifluoroacetic acid (TFA) containing 1 mM ATP followed by 10% acetonitrile in 0.1% TFA. Unlabelled and phosphorylated relaxin were then eluted with 80% acetonitrile in 0.1% TFA and fractions were analyzed by liquid scintillation counting in a Packard liquid scintillation analyzer M-1500 (Packard Instruments Company, Downers Grove, IL., U. S. A.). The volume of pooled eluates containing radioactivity was reduced in a SpeedVac concentrator (Savant Instruments, Inc., Farmingdale, N. Y.) to 1 ml then injected into a polyCAT A column (from PolyLC Incorporated, Columbia, Maryland, U. S. A.), equilibrated with buffer 1 (25% acetonitrile in 50 mM NaH₂PO₄ pH 7) using a Beckman HPLC system. The column was eluted with a linear gradient of 0-0.5 M NaCl in buffer 1. Fractions with high specific activity were collected and stored at -80 °C in the presence of 0.5% (v/v) BSA, 1.0 mM PMSF and 10 µg/ml leupeptin. All labelled relaxins were used within 1 week of preparation. The amount of relaxin H2 recovered from these fractions was determined by ELISA using a kit with antibody specific for human relaxin H2 generously provided by Genentech Incorporated, South San Francisco, California and the specific activity was calculated at 1275 Ci/mmol. The antibody used here for the ELISA could not detect relaxin H1, hence the amount of recovered labelled H1 peptide was quantitated based on the radioactive counts (cpm) obtained and the assumption that its loss on purification was equivalent to that measured for relaxin H2.

Optimization of conditions for relaxin binding.

To optimize the amount of membrane for the binding of relaxin H2 labelled with ³²P, concentrations ranging from 100 -500 µg/250 µl reaction volume were incubated with the label (50,000 cpm/tube or 63 pM) in the presence or absence of a 300-fold excess of unlabelled relaxin H2 for 30 min at 27 °C with gentle shaking. Optimum pH was

determined by incubating 250 µg of the membrane proteins in binding buffer over the pH ranges 4.5- 9.0. The time of incubation was studied over a 5- 360 min period and temperatures of 4 °C, 27 °C and 37 °C. The effect of divalent cations on relaxin binding was determined at 27 °C for 60 min, using a range of concentrations of CaCl₂, MgCl₂ and MnCl₂ with 250 µg membrane protein with and without competing unlabelled relaxin.

The "off rate" of ³²P-relaxin bound to the particulate membrane fractions was measured by the addition of a 100-fold excess of unlabelled relaxin H2 (6.3 nM) to the reaction mixture after a 60 min incubation of the membrane proteins with the ³²P-relaxin H2 at 27 °C. Aliquots of 250 µl were then taken at 0, 5, 10, 15, 30, 60, and 120 minutes and the binding compared with the reaction mixture for the equivalent time where no unlabelled relaxin was added.

Relaxin (labelled or unlabelled) readily adsorbs nonspecifically to any surface glass or plastic (Greenwood *et al.* 1981), to minimize this adsorption, all tubes and pipette tips were siliconized prior to use. In addition the reaction mixtures (at most 1.5 ml) were prepared in 2 ml siliconized microfuge tubes then aliquots of 250 µl were taken, incubated with gentle shaking, and bound complex then analyzed. The order of materials added into the reaction tubes was: 1mg/ml membrane proteins suspended in buffer D, unlabelled relaxin and labelled relaxin.

Freshly prepared membrane proteins were used for the assays since even flash freezing in liquid nitrogen and storage at -80 °C for up to two weeks caused a 25% decrease in binding. Precautionary measures were also taken during the course of this study to minimize the nonspecific adsorption of relaxin. With an ELISA for human relaxin H2 it was possible to track the loss of about 50% of the hormone when the relaxin was left to stand in the tube at room temperature for just 30 min, hence we prepared very small aliquots (10 µl) of the relaxins to avoid multiple freeze-thawing which were then stored at -80 °C and thawed on ice just before use. Tapping the tubes was avoided after thawing

the hormones since this increased the surface area for adsorption. Unlabelled relaxin was diluted by adding membrane proteins into small aliquot tubes and then serially diluting in tubes already containing membrane proteins, while labelled relaxin was suspended in buffer containing 0.5% BSA.

All the optimization studies were performed with the entire fetal membranes (amnion and chorion) and the adhering decidua parietalis. To determine the relative contributions of these layers to relaxin binding, particulate membrane fractions were prepared from each layer. The amnion was separated from the choriodecidua by delamination, and the chorion was separated from the adherent decidual tissues by scraping with a spatula. Membrane proteins were prepared from two patients for two separate binding assays. Each assay was performed with 6 replicates.

Measurement of specific binding of γ - ^{32}P -labeled relaxin H2 .

To triplicate or quadruplicate aliquots of membrane-enriched fraction in 250 μl binding buffer D (final concentration of $1\mu\text{g}/\mu\text{l}$) 63 pM of ^{32}P -labelled relaxin was added in the presence or absence of 6.3 nM unlabelled relaxin H2 . In all assays the bound complex was precipitated by the addition of 62.5 μl of 40% polyethylene glycol (PEG 8,000; for a 10% final concentration) and 12.5 μl of 20% bovine gamma globulin (for a 1% final concentration; Sigma Chemical Company, St. Louis, MO, U. S. A.), after incubation at 27 $^{\circ}\text{C}$ for 60 min, vortexed vigorously and incubated on ice for a further 10 min. It was then centrifuged at 40,000 g for 5 min at 4 $^{\circ}\text{C}$ in an Eppendorf microcentrifuge, the supernatants were immediately transferred into scintillation vials, while the pellets were resuspended and washed in ice cold binding buffer. They were then recentrifuged for 5 min at 40,000g at 4 $^{\circ}\text{C}$ rinsed 3x in ice cold PBS, recentrifuged to remove all excess liquid, and dissolved in scintillation cocktail for counting as described above. Binding

reactions were also performed in the absence of membrane particulate proteins to correct for non-specific precipitation of labelled peptides due to the addition of PEG. PEG(10%) when added to each assay tube in the absence of particulate membrane fractions gave only a 0.02% non-specific precipitation, which was considered to be insignificant. The difference between binding in the presence and absence of excess unlabelled relaxin H2 was taken as specific binding. Analysis of binding and affinity were performed using the Ligand program developed by Munson & Rodbard, 1980.

D. Results

Characteristics of ^{32}P -labelled relaxin H2 binding on particulate membrane-enriched fractions from human fetal membranes.

The absolute value for the specific binding obtained during the course of these studies varied considerably because human tissues used were from patients considered at term (38-40 weeks gestation) and undergoing elective Cesarean section prior to the onset of spontaneous labour. A total of 36 fetal membranes were used to prepare particulate membrane fractions over a period of 9 months and a range of 7- 28% specific binding of relaxin to these particulate membrane fractions was obtained. The specific binding of ^{32}P -labelled relaxin H2 was essentially linear with increasing concentration of membrane protein over a range of 100-250 μg , thereafter it showed no further increase even when a higher concentration of PEG was added. The optimal amount of binding occurred at 250 μg protein and this concentration was used in all subsequent assays. With this concentration of particulate membrane preparation and increasing concentrations of labelled relaxin, the amount of bound label to particulate preparation increased specifically and saturation of binding was achieved at about 600 pM.

There was a narrow pH optimum (pH 7-8) for the binding of ^{32}P -labelled relaxin H2, the maximum was obtained at pH 7.5, therefore this was used in all further experiments. A time course for relaxin H2 binding was conducted (Fig. 1a) and maximum binding occurred at 60 min at 4 °C and 27 °C, and after 20 min at 37 °C, however at this temperature only 66% of the maximum possible binding was achieved. There was an increase in non-specific binding with increased incubation time at all temperatures and it was most pronounced at 37 °C. The binding at 27 °C was only a fraction better than at 4 °C, although at both temperatures binding remained high for a further 60 min. From these curves the conditions for the assays were optimized so for all subsequent studies the binding at 27 °C for 60 min at pH 7.5 was used.

The addition of divalent cations affected the binding of ^{32}P -labelled relaxin H2 to the particulate membrane preparations (Fig. 1b). The effect of Ca^{2+} was greatest at a lower concentrations (1.5 mM) than with either Mg^{2+} or Mn^{2+} (2.5 mM). However, the addition of either of the latter ions had a slightly greater effect than Ca^{2+} alone. Based on these results Mn^{2+} and Mg^{2+} ions were added in binding buffers at a final concentration of 2.0 mM just before each of the binding experiments.

The "off rate" of ^{32}P -labelled relaxin H2 was studied by the addition of excess unlabelled relaxin H2 at binding equilibrium, this led to a rapid decrease in specific binding. After 60 min approximately 90% of bound ^{32}P -labelled relaxin H2 has dissociated. Incubation for another 60 min did not result in total dissociation and at least 5% of labelled relaxin is either irreversibly bound or only slowly dissociable (data not shown). Membrane particulate fractions from separated fetal membrane layers showed that binding activities were mainly from the chorion and decidua (27.8% and 30.2% specific binding, respectively) with very low binding in the amnion (4.8% specific binding). Based on these results subsequent assays were conducted using amnion, chorion and decidua together, since amnion constitutes only a very minor component of the tissue.

Binding specificity and affinity of human relaxins.

The specificity of relaxin binding to particulate membrane-enriched fractions was investigated using a number of different relaxins and recombinant human insulin for competition. A range of concentrations of these hormones was allowed to bind to 250 μg particulate preparations for 30 min at 4 $^{\circ}\text{C}$, labelled relaxin H2 (63 pM) was then added and further incubated at 27 $^{\circ}\text{C}$ for 60 min. The amount of unlabelled ligand needed to inhibit 50% (IC_{50}) of the binding of labelled relaxin H2 was determined from the displacement curves (Fig. 2). These curves were generated by the Ligand program (Munson & Rodbard, 1980). The IC_{50} of human relaxins H2 and H1 were 28 pM and 60 pM, respectively. Porcine relaxin was calculated to have an IC_{50} ten-fold higher than human relaxin H2 (280 pM), while that for guinea pig relaxin could not be calculated since it required 600 nM to inhibit approximately 10% of maximal specific binding. Human recombinant insulin failed to displace the binding of relaxin even at a very high concentration of 600 nM.

Labelled relaxin H2 or H1 were used as ligands and was each displaced by a range of concentrations of unlabelled relaxins H1 and H2; qualitatively similar displacement curves were obtained (Fig 3a and 3b respectively). However, labelled relaxin H2 was less readily displaced than the relaxin H1 label with the same concentration of unlabelled relaxin H1 and H2. With either of the labelled ligands unlabelled relaxin H2 showed a 1.5 - 2 fold better displacement than unlabelled relaxin H1. The IC_{50} for unlabelled relaxin H1 and H2 with relaxins H2 and H1 as labelled ligand was 6 pM, 4 pM, 20 pM and 28 pM, respectively. Based on these curves the affinity constant $K_a = 4.6 - 5.1 \times 10^{-8}$ M for relaxin H1 and $K_a = 1.9 - 2.2 \times 10^{-9}$ M for relaxin H2 were obtained. A Scatchard analysis employing the same Ligand program on increasing concentration of labelled

relaxin (concentration range of 0.63 -25.6 pM) H2 showed that the best fit model is for a single class of binding sites (data not shown) with a $K_a = 2.15 \times 10^{-9} \text{M}$.

E. Discussion

Relaxin is a product of the human choriodecidua (Sakbun et al. 1990) and we have proposed it to be a local modulator of collagenolysis in the fetal membranes (Bryant-Greenwood, 1991). Over the last 8 weeks of pregnancy when there is a need for the membranes to adjust to the rapidly increasing volume of the uterus, there is significant decrease in the total collagen content of the amnion (Skinner *et al.* 1981). Relaxin causes the release of plasminogen activator and collagenolytic activity from amnion and chorion cells *in vitro* (Koay *et al.* 1983). A relationship has recently been shown between the expression of the genes for relaxin and interstitial collagenase in the choriodecidua during the peripartal period (Bryant-Greenwood & Yamamoto, 1995).

This paper is the first study on the characterization of the binding of the two human relaxin peptides H1 and H2 *in vitro* to target tissues for relaxins. The results confirm the general approach of Mercado-Simmen *et al.* (1980) that a relaxin receptor can be identified with an appropriately labelled relaxin and the specific findings of Koay *et al.* (1986) that a relaxin receptor is present in the human fetal membranes since the addition of relaxin to these tissues *in vitro* has a biological effect.

From the results presented here it appears that the binding of the human relaxins fit the classical criteria for a "true" receptor (De Meyts, 1981). Binding of labelled relaxin H2 was shown to be saturable, reversible, reproducible, pH, time- temperature- and cation-dependent in a tissue shown to be biologically responsive to added relaxin. Mercado-Simmen *et al* (1980) reported that manganese ions had the most effect on relaxin binding on crude membranes prepared from rat uterus. Our results showed that both manganese and magnesium ions at a 2.5 mM concentration enhanced relaxin binding. The roles of these cations in relaxin binding are not clear at present, but may increase the affinity and the concentration of relaxin binding sites (Pearlmutter & Soloff, 1979). Separation of the fetal membrane layers and dissection from the decidua have shown that binding is essentially equal between the chorion and decidua and very low in the amnion. The co-existence of specific binding for relaxins in the choriodecidua with relaxin gene expression and immunological detection of relaxin peptide(s) implies an autocrine action within these tissues or paracrine actions between the different cell types in the chorion and decidua, respectively.

Saturation and displacement studies analysed by the Ligand program (Munson & Rodbard, 1980) gave the best fit model for a single class of relaxin binding sites with an affinity constant (K_a) of 1.9 - 2.2 nM, in contrast to that reported by Mercado-Simmen *et al*. (1980) and Koay *et al* (1986) where a biphasic mode for relaxin and its receptor were proposed in the rat uterus and human fetal membranes, respectively.

These differences may be due to the different labelling and homologous peptides used in our assays. Our results however, are very similar to that obtained by Yang *et al* (1992) where they reported a single class of relaxin binding sites on mouse uterine ($K_a = 1.96$ nM) or brain ($K_a = 1.57$ nM) crude membrane preparations using a monocomponent ^{125}I -porcine relaxin as the ligand. Osheroff *et al* (1992) also reported a single class of relaxin binding sites obtained from autoradiography of tissue sections of rat uterus ($K_a = 1.3$ nM) brain ($K_a = 1.4$ nM) and heart atrium ($K_a = 1.4$ nM), using ^{32}P -labelled human relaxin H2.

The binding of labelled relaxin H2 to particulate membrane fractions from the human fetal membrane is highly specific, and could only be displaced by unlabelled relaxin in the order of H2 \geq H1 > porcine \gg guinea pig relaxin. The ability of porcine relaxin to bind to the human relaxin receptor is consistent with the observations that porcine relaxin can stimulate collagenase activity in cultured cells from human amnion and chorion (Koay *et al.* 1983), increase cAMP levels in cultured endometrial glandular epithelium (Chen *et al.* 1988) and enhance mRNA synthesis of prolactin and insulin-like growth factor binding protein-1 in human endometrial/decidual cells (Tseng *et al.* 1992). Porcine relaxin however, requires a ten-fold higher concentration than relaxin H2 to inhibit relaxin H2 binding to its receptor. This suggests a requirement for a specific conformation or configuration of the relaxin to be able to fit properly into the receptor binding site. The biological activity of guinea pig relaxin *in vitro* nor *in vivo* has not been demonstrated, however it is known to have a high biological specificity.

While human relaxin H2 and native porcine relaxin were equipotent in the mouse pubic symphysis assay (Lucas *et al.* 1989), guinea pig relaxin is active only in the homologous guinea pig pubic symphysis bioassay (Bryant-Greenwood & Schwabe, 1994). It is possible that the guinea pig relaxin has a secondary structure that fits well into the guinea pig but not in the human receptor binding site hence the low binding activity in the fetal membrane assay. This species specificity of the relaxins is unusual and as yet appears not to follow any particular pattern (Bryant-Greenwood & Schwabe, 1994). Human recombinant insulin was inactive, as would be expected despite its general structural similarity to relaxin (Crawford *et al.* 1984; Eigenbrot *et al.* 1991).

Of greater interest was the comparison between the two homologous relaxin peptides H1 and H2 with 77% identity in the amino acid sequences of these fully processed, mature peptides (Bryant-Greenwood & Schwabe, 1994) and they very likely share a common receptor. When labelled relaxin H2 or labelled relaxin H1 were used as ligands the shape of the displacement curves for these two peptides were similar however, relaxin H1 showed a 5-fold lower affinity for this receptor compared with relaxin H2 and suggests a preferential binding of relaxin H2 over relaxin H1. Although both relaxin H1 and H2 genes are expressed in the choriodecidua and relaxin- immunostaining has been demonstrated, it is not known whether this is attributable to relaxin H1 or H2.

Further work is required to elucidate the developmental regulation of the two expressed human relaxin genes in the fetal membranes/decidua, whether both peptides are translated and whether the relaxin peptides and their receptors are coregulated.

Meanwhile the development here of a specific binding assay for the relaxins will assist our current work on the molecular cloning of the relaxin receptor(s) and may allow us to understand more precisely the receptor-ligand interactions of these hormones.

F. Acknowledgments

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FIGURE 1. (a) Time-course of labelled relaxin binding. (b). Effect of divalent cations.

Graphs are representatives of at least two separate experiments and each point is the mean of at least three determinations.

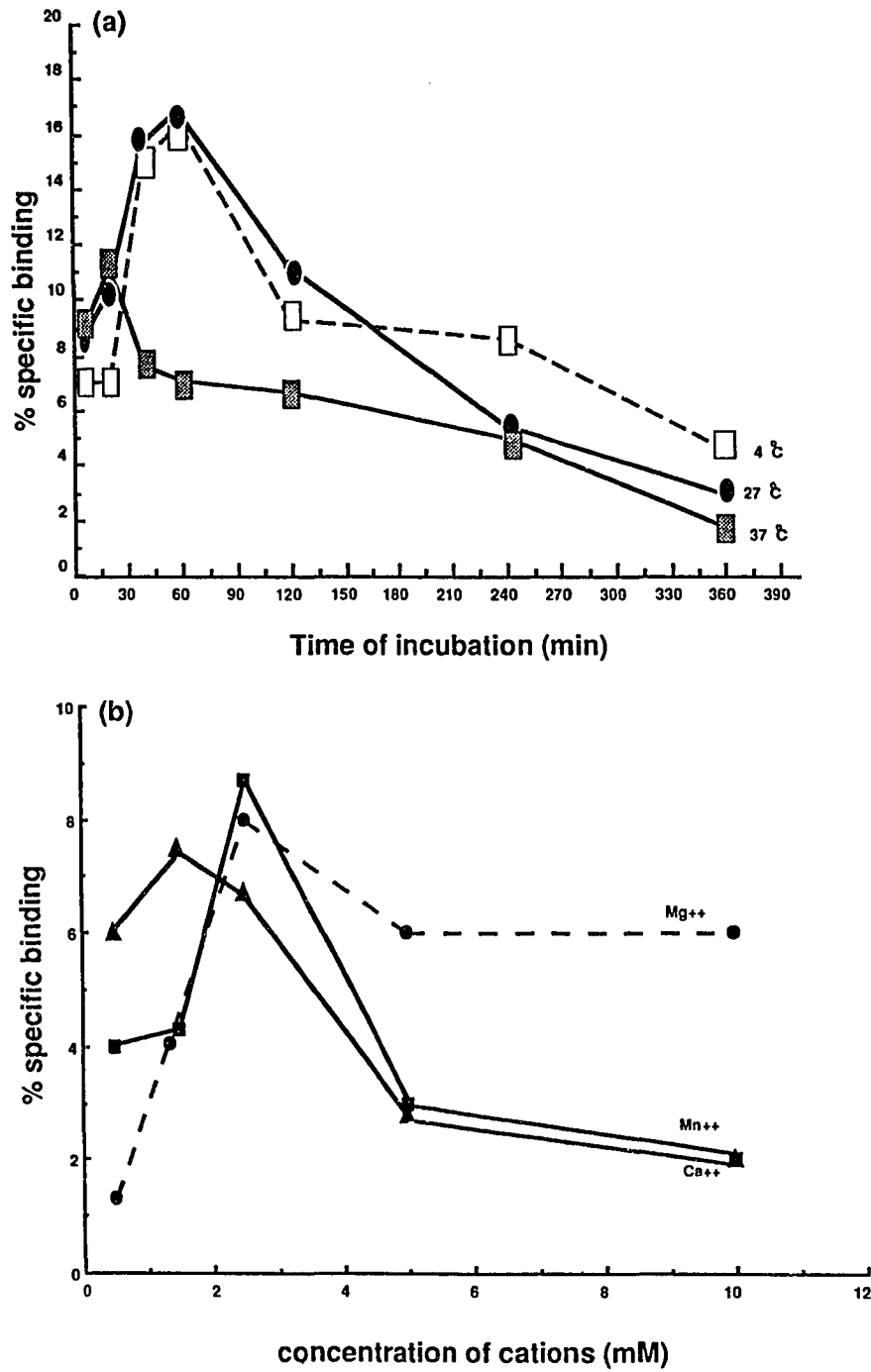
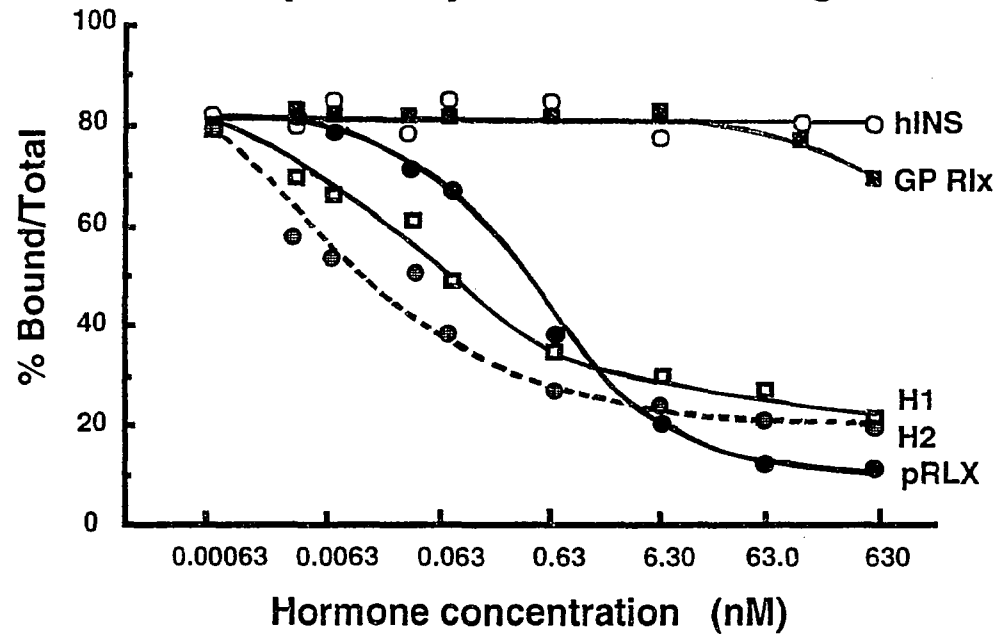
Garibay-Tupas et al.
Figure 1

FIGURE 2. The specificity of labelled relaxin H2 binding determined by addition of unlabelled relaxins and insulin. Quadruplicate samples of membrane-enriched fractions (250 μ g/tube) were incubated with increasing concentrations of unlabelled human relaxin H2 (●), human relaxin H1 (○) porcine relaxin (D) guinea pig relaxin (■) and human recombinant insulin (O) for 30 min at 4 °C prior to the addition of 63 pM of labelled relaxin H2, incubated for a further 60 min at 27 °C. Results are from a representative experiment.

Specificity of relaxin binding

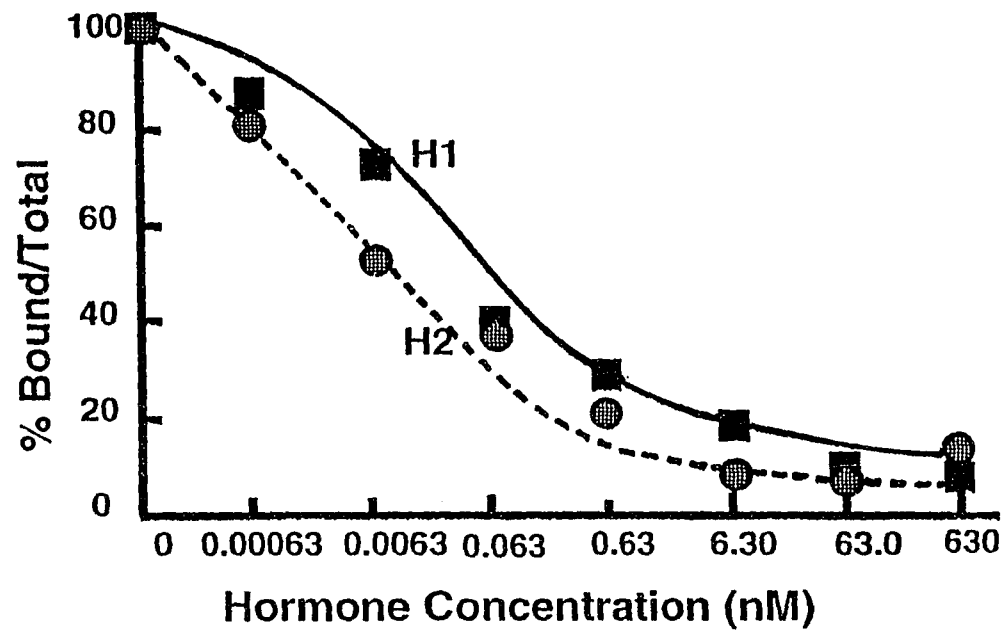


hINS = recombinant human insulin
 GP Rlx = guinea pig relaxin

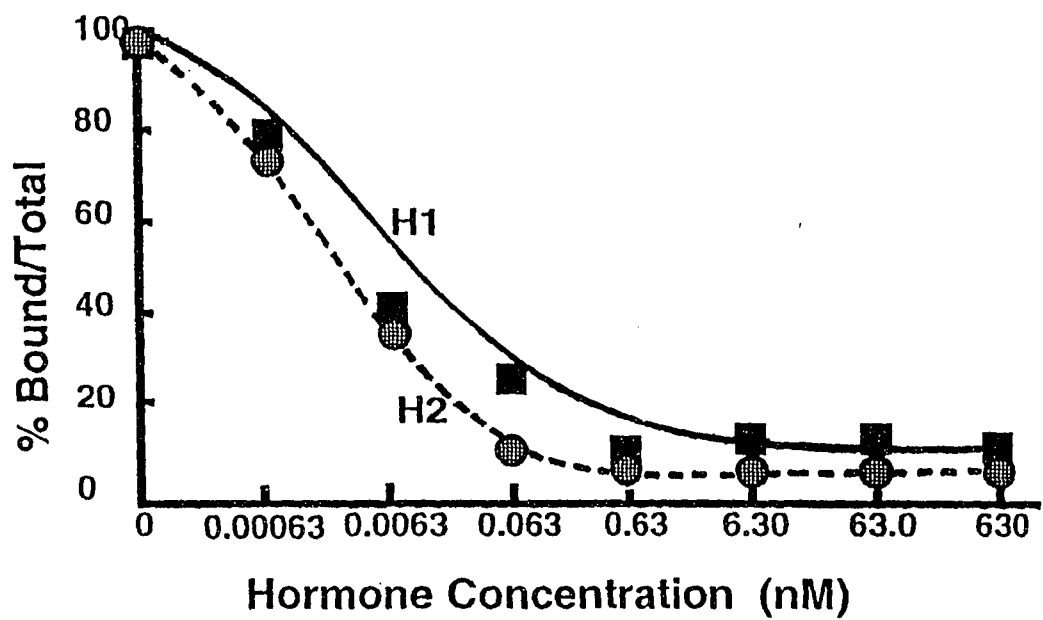
H1, H2 = human relaxin H1, H2
 pRLX = porcine relaxin

FIGURE 3. Inhibition of binding of (a) labelled relaxin H2 with increasing concentrations of unlabelled relaxins H1 and H2 (b) labelled relaxin H1 with increasing concentrations of unlabelled relaxins H1 and H2. Results are from a representative experiment.

(a) Inhibition of labeled relaxin H2 binding



(b) Inhibition of labeled relaxin H1 binding



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CHAPTER IV

DISCUSSION AND SUMMARY

A. The PRL autocrine/paracrine systems in the fetal membranes, decidua and placenta

Transcription and translation of human the PRL-R in the chorionic cytotrophoblast \geq decidua parietalis $>$ amniotic and placental syncytiotrophoblast has been demonstrated using Northern analysis, *in situ* hybridization and immunocytochemistry. These data unequivocally demonstrate that PRL is an autocrine/paracrine hormone in these tissues. The wide distribution of the PRL-R in the fetal membranes, decidua and placenta suggests role(s) at the maternal-fetal interface during normal pregnancy and parturition. Most significantly, the increased transcription of the PRL-R after normal labor and delivery as measured by quantitative Northern analyses strongly suggests a role(s) for PRL in the peripartal period. The plethora of biological activities ascribed to PRL make the definition of the biological consequence of ligand receptor interaction at several intrauterine sites a difficult and important task for future work. However, two of the most plausible actions could be to serve as an immunocrine hormone within the decidua and to increase the release of collagenolytic activity from the amnion/chorion cells to modify the ECM of the fetal membranes.

The technique of western blot analysis allows separation of the PRL-R-related proteins produced at the different sites of translation. Thus, the classical 95-85 kDa membrane bound PRL-R was detected in addition to species of 63 <63, >30 and 30 kDa

in intact amnion, chorion and decidua (ACD) lysates. Tissue lysates showed the >30 kDa band as the major product in the decidua while the 63 and 30 kDa bands were predominant in the amnion and chorion. The transport of PRL is dependent on an intact amnion, chorion and decidua, which indicates presence of a mechanism(s) for PRL transport across these tissues depositing it into amniotic fluid, Fig. 20. The role(s) of the four molecular species related to the PRL-R and detected by western blotting supports but does not prove the existence of cell-specific processing of the PRL-R and the actions of one or more of these proteins as a PRL binding/transport protein.

The detection of a putative binding/transport protein for PRL in the fetal membranes and decidua is not unexpected given that decidual PRL, unlike pituitary PRL, is not stored in granules of these cells but is readily synthesized and secreted. On the other hand, the half-life of amniotic fluid PRL has been shown to be 4.2 hours in comparison to the 12-15 minute half-life of its pituitary counterpart (Tyson, *et al.*, 1974). This shorter half life in amniotic fluid may reflect its protection from degradation by a binding protein. However, further work is necessary with an antibody to the human PRL-R and to known epitopes of this protein (when they become available) to clarify the structure-function of the four immunoreactive PRL-R proteins detected in these studies.

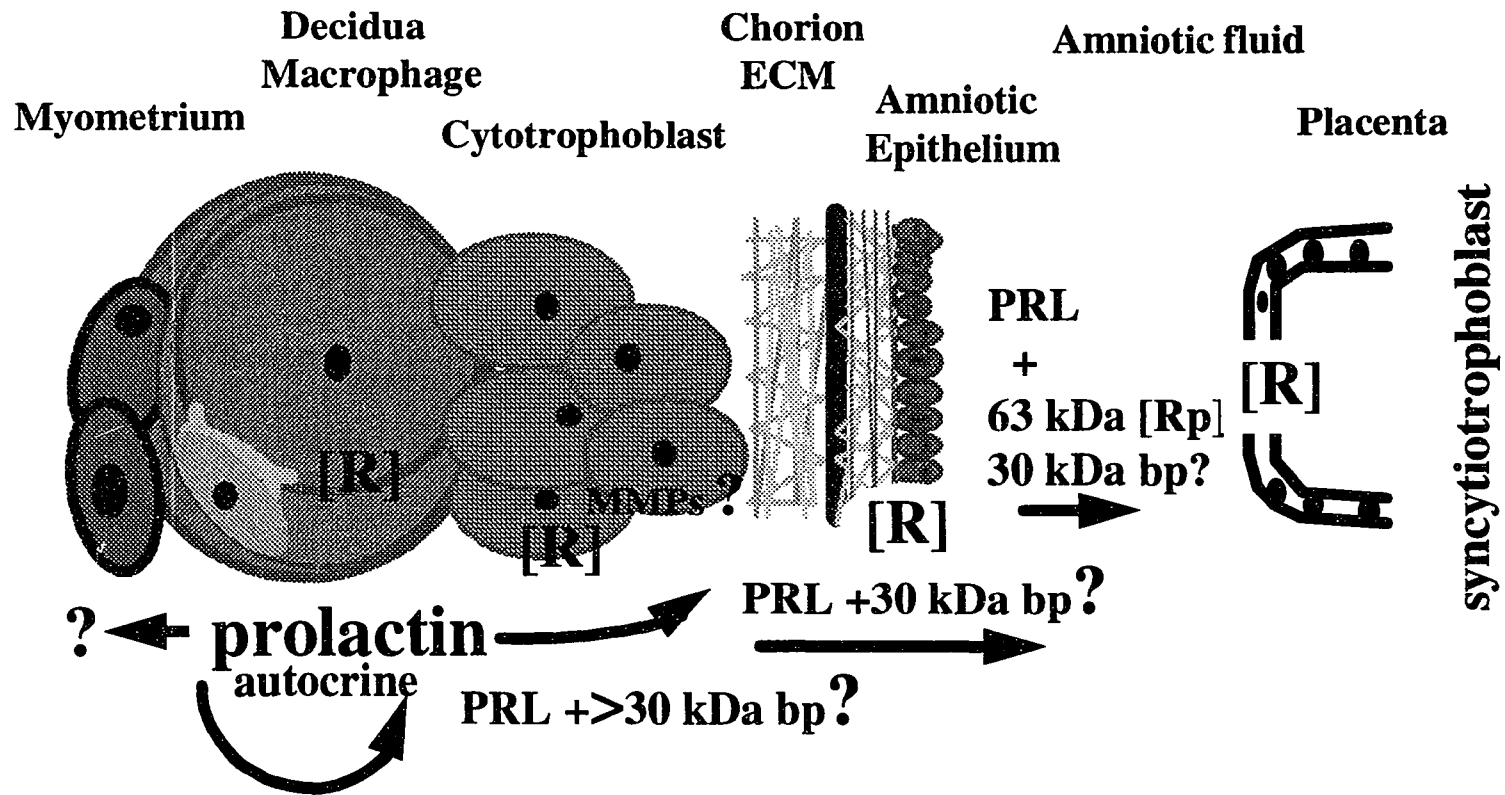


Figure 20. Schematic illustrating of prolactin and the prolactin receptor at the maternal fetal interface: PRL, prolactin; R, prolactin receptor; bp, binding protein; MMPs, matrixmetalloproteinases; ECM, extracellular matrix

B. The RLX autocrine/paracrine systems in the fetal membranes, decidua and placenta

The recent chemical synthesis of human RLXs H1 and H2 (Bullesbach and Schwabe, 1991) and a suitable method of labeling and purifying their products (Osheroff, et al., 1990), has made it possible to characterize the RLX-R in the human choriodecidua for the first time with homologous ligands. The presence of a RLX-R in human choriodecidua was previously suggested from work carried out by Koay *et al.*, 1986, who demonstrated the binding of labeled porcine RLX to particulate preparations of amnion and chorion cells as well as a biological response in response to the addition of exogenous porcine RLX.

Assay conditions had to be reoptimized for the binding of $\gamma^{32}\text{P}$ labeled RLX H2 to choriodecidua enriched membrane particulate fractions in the current studies and the binding shown to be saturable, and dependent on time and temperature. A single class of RLX binding site(s) with an affinity constant of 2.15 nM was demonstrated. There may be no requirement for a transport/binding protein for the RLXs, since these hormones are also produced by the placenta (unlike PRL). Thus once RLXs are secreted and/or bound to their receptor they are rapidly degraded, as suggested by the very low levels detected immunologically in amniotic fluid (Johnson, *et al.*, 1991).

The specificity of RLX binding to these particulate fractions was determined by competition using both synthetic human RLX H1 and H2, porcine RLX, guinea pig RLX

and recombinant human insulin to compete off labeled human RLX H2. The amount of unlabeled ligand needed to inhibit 50% (IC_{50}) of the binding of labeled RLX H2 was calculated from the displacement curves. The IC_{50} values for human RLX H2, H1 and porcine RLX were: 28pM, 60pM and 280pM respectively. Thus, the binding of labeled human RLX could be displaced by unlabeled RLX in the order of $H2 \geq H1 >$ porcine >>> guinea pig RLX. When these data were compared it was shown that RLXs H2 and H1 can readily displace the binding of either ^{32}P labeled human RLX H1 or H2, yielding almost identical displacement curves, supporting the view that a single RLX receptor target for both the human RLXs produced in these tissues. Human RLX H1 was also labeled with ^{32}P and its binding demonstrated for the first time. However, a five-fold lower affinity of RLX H1 for a receptor (compared to RLX H2) suggests a preferential binding of RLX H2 over RLX H1 in this tissue

This data, taken together with the coexpression of the human RLX H1 and H2 in the human decidua, provides further evidence that RLX is an autocrine/paracrine hormone in the decidua and fetal membranes. Figure 21 summarizes the distribution of RLX and its RLX-R at the maternal-fetal interface.

C. The Interaction between the PRL and RLX paracrine systems

Immunocytochemical studies have indicated that RLX is present in the same decidual cells as PRL (Bryant-Greenwood, *et al.*, 1987). The synthesis and release of decidual PRL and RLX appear to be locally regulated by autocrine/paracrine factors produced within the fetal membranes, decidua and placenta, and a possible relationship

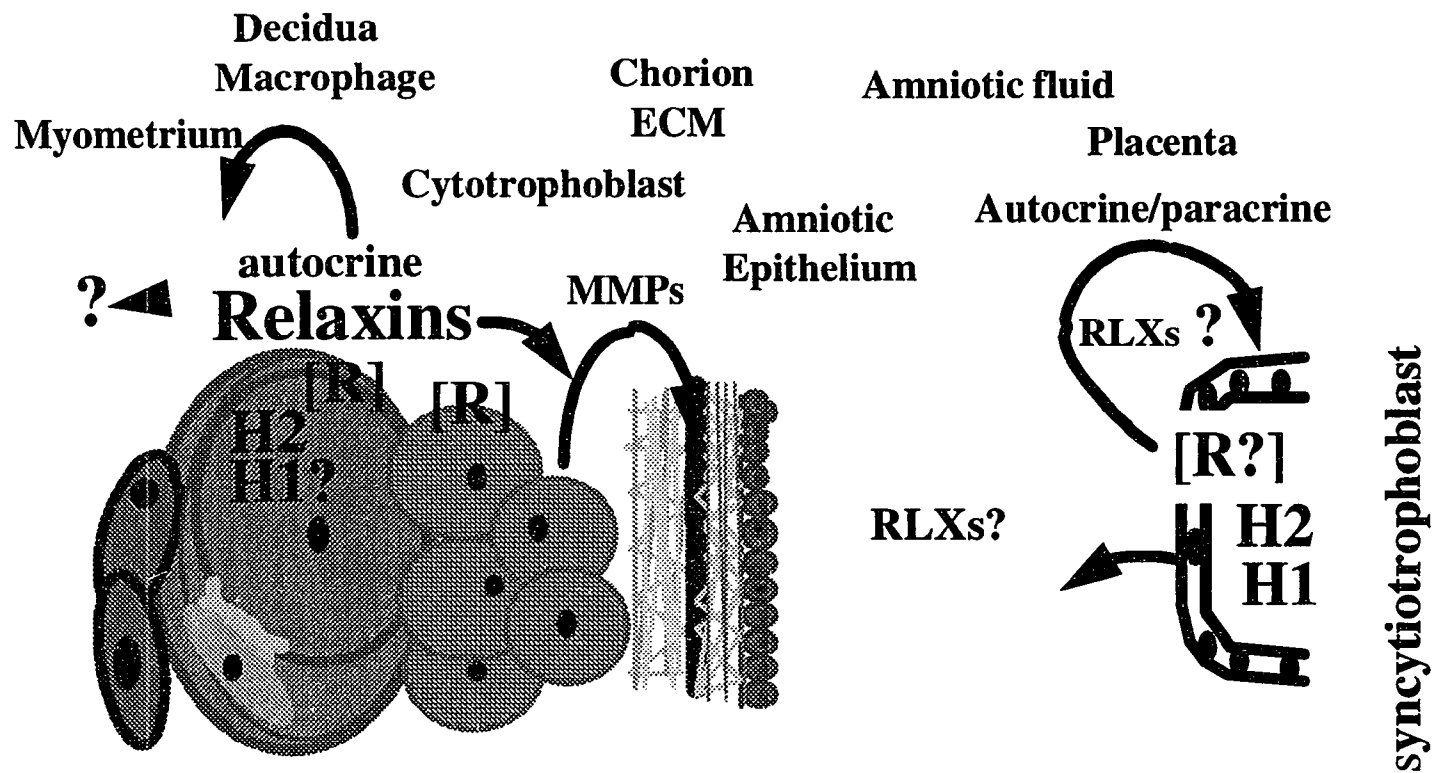


Figure 21. Schematic illustrating of relaxin and the relaxin receptor at the maternal fetal interface: RLX, relaxin; R, relaxin receptor; MMPs, matrix metalloproteinases; ECM, extracellular matrix

between them has been suggested from in vitro (Huang et al., 1992), and in vivo studies (Bryant-Greenwood, et al., 1993).

Exogenous addition of PRL or RLX in vitro cause increase in the release of collagenolytic activities from fetal membrane cells into the surrounding media (Koay, et al., 1986). More recent work has shown that RLX H2 can cause a dose dependent increase in specific metalloproteinases from fetal membrane explants (Qin, et al. 1995). Preliminary data (to be published) suggests that PRL may augment and/or complement the role of RLX in releasing the enzymes from these tissues.

Receptors for both PRL and RLX have been demonstrated in the decidua itself, showing their autocrine/paracrine modes in this tissue and in the chorion showing paracrine function. The decidua is heterogeneous and further work is required in order to define the cell types producing each of these hormones. While the stromal decidual cells themselves may possess receptors for each of these hormones, receptors on the other cells present in the decidual layer, e.g. lymphocytes or macrophages, would link the endocrine and immune systems in these tissues.

An increase in PRL-R transcription has been demonstrated after normal spontaneous labor and delivery. Such changes in the RLX receptor over this peripartum period were not yet sought, but would be important to further define its activities in relationship to parturition. The distribution of both PRL and RLX receptors in the fetal membranes decidua and placenta are summarized in fig. 22.

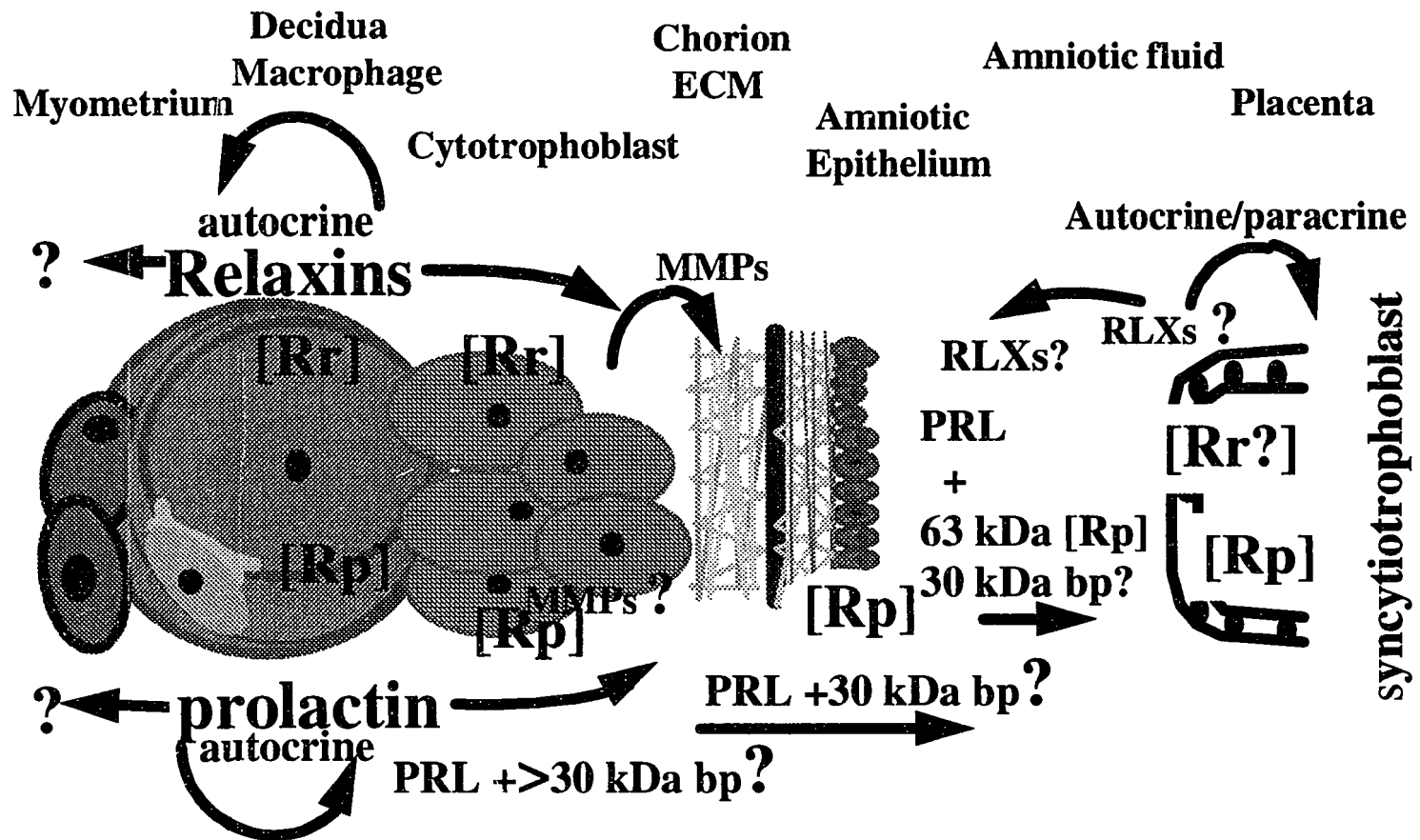


Figure 22. Schematic illustrating of prolactin and relaxin at the maternal fetal interface: Rr, relaxin receptor; Rp, prolactin receptor; bp, binding protein; PRL, prolactin; RLX, relaxin; MMPs, matrix metalloproteinases; ECM, extracellular matrix

CHAPTER V BIBLIOGRAPHY

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